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EPSTEIN-BARR VIRUS-RELATED B CELL LYMPHOPROLIFERATIVE DISORDER AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION

Presentata da: Dott.ssa Angela Chiereghin

Coordinatore Dottorato

Chiar.mo Prof. Lucio Cocco

Relatore

Chiar.ma Prof.ssa Tiziana Lazzarotto

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List of abbreviations

β-TM: beta-thalassemia major

AA: amino acid

ALCL: anaplastic large cell lymphoma

ALL: acute lymphoblastic leukemia

Allo-HSCT: allogeneic hematopoietic stem cell transplantation

AML: acute myeloid leukemia

ATG: antithymocyte globulin

Auto-HSCT: autologous hematopoietic stem cell transplantation

BCR: B cell receptor

BL: Burkitt lymphoma

BM: bone marrow

CAEBV: chronic active EBV

CHL: classical Hodgkin lymphoma

cIL: cellular interleukin

CMI: cell-mediated immunity

CML: chronic myeloid leukemia

CTLs: cytotoxic T cells

D/R: Donor/Recipient

DAMPs: damage-associated molecular patterns

DLBLC: diffuse large B-cell lymphoma

E: early

EA-D: diffuse early antigens

EA-R: restricted early antigens

EAs: early antigens

EBER: EBV encoded small RNA

EBNA: Epstein-Barr virus nuclear antigen

EBNA-LP: Epstein-Barr virus nuclear antigen leader protein

EBV: Epstein-Barr virus

EliSpot: Enzyme-linked ImmunoSPOT

g: glycoprotein

GC: germinal center

GITMO: Italian Group of Bone Marrow Transplantation

gp: glycoprotein

GVHD: graft-versus-host disease

GVL: graft-versus-leukemia

Gy: grays

HHV4: human herpesvirus 4

HHV8: human herpesvirus 8

HL: Hodgkin lymphoma

HLA: human leukocyte antigen

HLA-DR: human leukocyte antigen - antigen D Related

HSC: hematopoietic stem cell

HSCT: hematopoietic stem cell transplantation

IE: immediate-early

IFN: Interferon

Ig: Immunoglobulin

IL: interleukin

IM: infectious mononucleosis

IR: internal repeat

L: late

LLQ: lower limit of quantification

LMP: latent membrane proteins

MA: myeloablative

MDS: myelodysplastic syndrome

MHC: major histocompatibility complex

miRNAs: microRNAs

MM: multiple myeloma

MS: myeloid sarcoma

MUD: matched unrelated donor

NF-kB: nuclear factor kappa B

NHL: non-Hodgkin lymphoma

NK: Natural Killer

NMA: nonmyeloablative

NPC: nasopharyngeal carcinoma

Ori-lyt: lytic origin of replication

Ori-P: origin of plasmid replication

PAMPs: pathogen- associated molecular patterns

PB: peripheral blood

PBMCs: peripheral blood mononuclear cells

PTLD: post-transplant lymphoproliferative disorder

RIC: reduced intensity conditioning

SAA: severe aplastic anemia

SFCs: spot forming cells

TBI: total body irradiation

TERT: telomerase reverse transcriptase

TR: terminal repeat

UCB: umbilical cord blood

UL: unique long

US: unique short

VCA: viral capsid antigen

vIL: viral interleukin

WB: whole blood

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Part I

INTRODUCTION

1

EPSTEIN-BARR VIRUS

Epstein-Barr virus (EBV) was first discovered in a Burkitt lymphoma (BL)-derived cell line by Michael Epstein, Yvonne Barr and Bert Achong in 1964 by electron microscopy (1). EBV or human herpesvirus 4 (HHV4), is a double-stranded DNA virus that belongs to the Herpesviridae family and Gammaherpesvirinae subfamily. The latter one includes another important human gammaherpesvirus such as human herpesvirus 8 (HHV8) also known as Kaposi's sarcoma-associated herpes virus. These two viruses are characterized by establishing latent infection in lymphocytes, restricted host range for infection in cell culture, episome persistence in dividing cells and oncogenic associations (2).

EPIDEMIOLOGY

Although herpes viruses are ubiquitous in nature, humans serve as the only natural host for EBV. EBV is a ubiquitous γ-herpes virus with tropism for B lymphocytes that infects at least 90% of the population worldwide in which establishes a life-long asymptomatic infection (3). Two EBV types circulate within the population, EBV-1 and EBV-2 (also named types A and B) and are different in geographic distributions and in the organization of the genes encoding EBV nuclear antigen (EBNA). EBV-1 is more prevalent in most populations and type 1 EBV isolates are more efficient in trans-forming infected-B cells *in vitro* than type 2 isolates, although differences in EBV types have not correlated with human diseases and both types co-exist in all populations that have been studied. EBV-2 is detected frequently in New Guinea, equatorial Africa, and Alaska (2,4). Infection is most common among children of 2 and 4 years of age and young adults of 15 years of age. Primary infection with EBV in infants and young children is usually symptomatic or results in non-specific symptoms. In developing countries, infants and young children are infected with EBV and infectious mononucleosis (IM) is much less common. For example, in areas of Africa where BL is common, 50% of children are infected with EBV before 1 year of age. Whereas, in developed countries, about 50% of adolescents and young adults are EBV seronegative, and about 25% of these seronegative persons who become infected with EBV develop

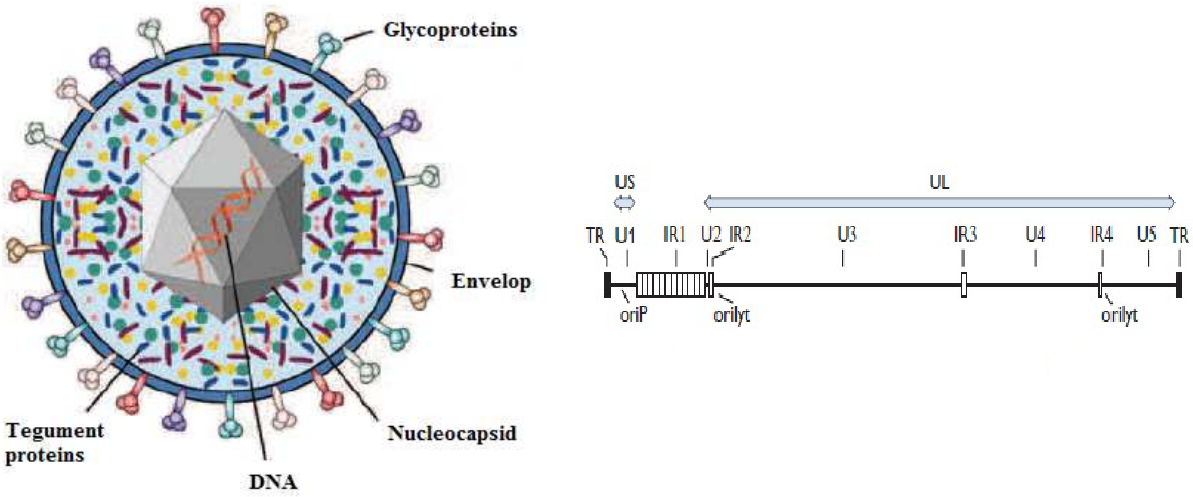
IM. EBV is usually spread by infected saliva and some observations suggest that the virus may be spread by sexual intercourse. In fact, EBV was detected in both male and female genital secretions, but the level of EBV in genital secretions is lower than in saliva. Finally, EBV can be acquired from blood transfusions or from receipt of allogeneic donor stem cells or tissue (5).

VIRUS AND GENOME STRUCTURE

EBV has a toroid-shaped DNA core in a nucleocapsid with 162 capsomeres, an outer envelope with external glycoprotein spikes, and a protein tegument between the nucleocapsid and envelope (Figure 1a). Among the viral glycoproteins, gp350, gH, gB, gp42, gp220 and gL are those important for binding and subsequent fusion of the virion envelope with cellular membranes (2). The EBV genome is a linear, double-stranded DNA of approximately 172 kb, encoding approximately 100 viral proteins (4). It contains five unique sequence elements (U1 to U5) interspersed with one major internal repeat (IR1) and three smaller internal repeats (IR2 to IR4). The major internal repeat element contains six to twelve tandem repeats of 3 kb, depending on the virus strain. There are three replication origins: two of these (Ori-lyt, lytic origin of replication) are used during productive infection and one (Ori-P, origin of plasmid replication) is used during latent infection. At both termini of the linear genome, there are variable numbers of 0.5-kb tandem repeats; EBV DNA circularizes upon entering the cell via a terminal repeats (TR) sequence (6). The Figure 1b depicts the linear EBV genome.

Figure 1: Schematic depictions of the: **a)** EBV virion and **b)** overall EBV genome arrangement with the unique short (US) and unique long (UL) regions shown. (Adapted from Fields. Virology. 6th Edition)

a) **b)**

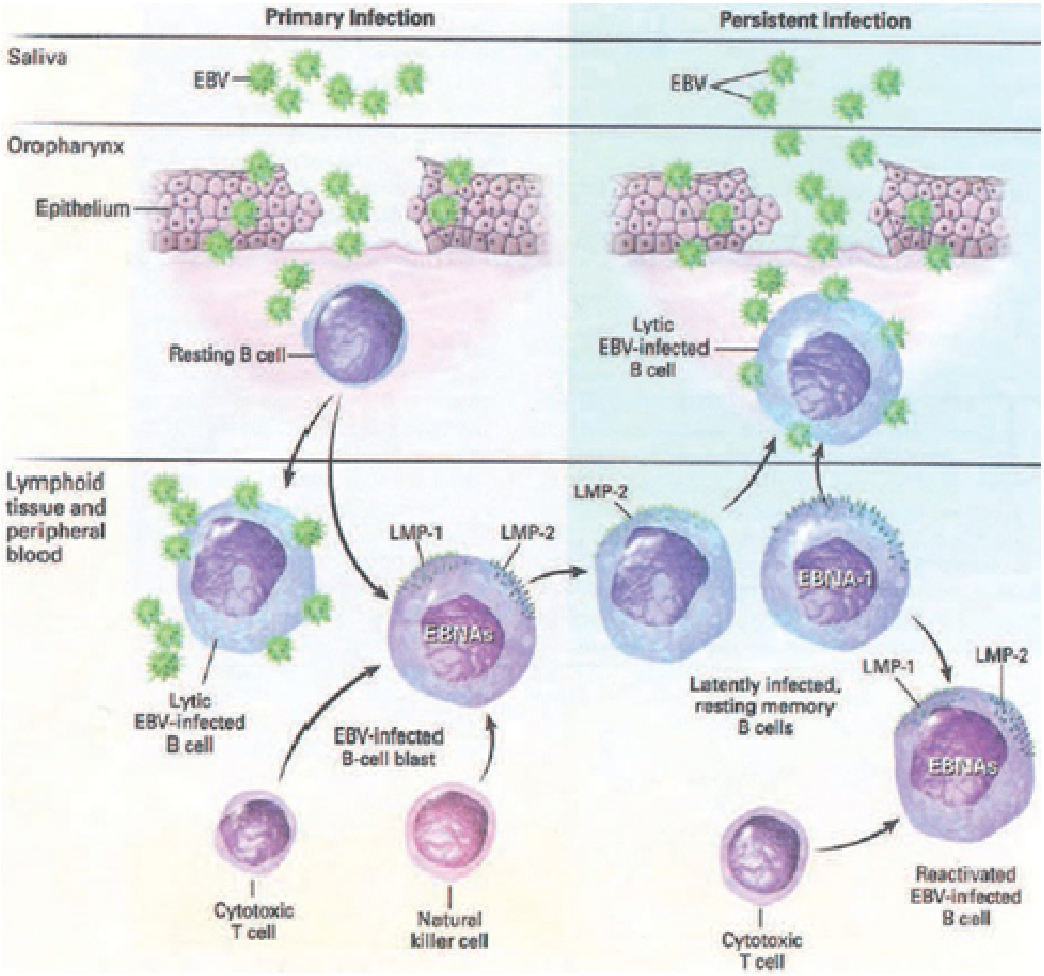


EBV LIFE CYCLE

Almost all EBV-seropositive hosts shed virus in the saliva, and infection occurs when an EBV-naïve person is exposed to EBV-rich saliva. Oropharyngeal epithelium is an entry point for EBV via the immunoglobulin (Ig) A-EBV complex to IgA receptors on the epithelium, and active viral replication - lytic infection - occurs there. The virus spreads out from the infected cells and directly infects nearby B lymphocytes via viral enveloping of gp350 to the B lymphocyte surface molecule CD21. Penetration of the virus requires interaction between viral gp42, which makes a complex with gH and gL, and human leukocyte antigen - antigen D Related (HLA-DR) on B lymphocytes. Indirectly, the infected epithelium can transmit the virus to B lymphocytes (7). EBV-infected B cells in the lymphoid tissues and blood undergo lytic infection or become B cell blasts, which are controlled in immunocompetent individuals by Natural Killer (NK) cells and cytotoxic T cells. Latently infected resting memory B cells express no viral proteins unless they divide; if they reactivate, they are controlled by cytotoxic T

cells. Some latently infected cells traffic to the oropharynx where they undergo lytic replication and release the virus (Figure 2) (2).

Figure 2: Representation of EBV life cycle. (From Fields. Virology. 6th Edition)



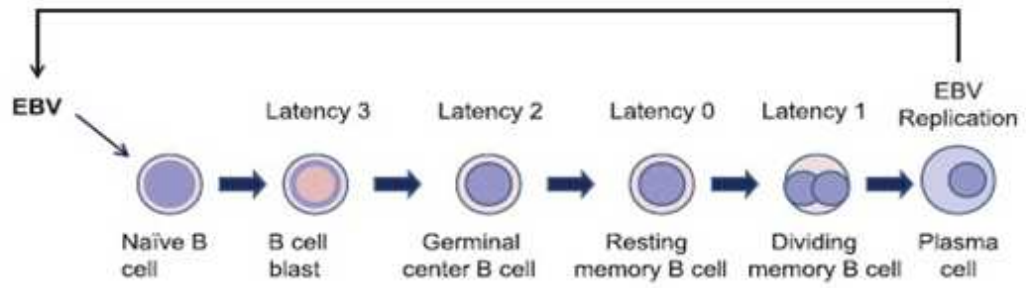
LATENT EBV INFECTION

Only a handful of genes are expressed during latent infection: six EBV nuclear antigens (EBNA-1, -2, -3A, -3B, -3C and -LP leader protein), three latent membrane proteins (LMP-1, -2A and -2B) and two small, untranslated RNAs that are transcribed by RNA polymerase III (EBV encoded small RNA (EBER)-1 and -2). EBNA-1 binds to viral DNA and maintain its episomal form (7). EBNA-2 and EBNA-LP together are responsible for transcriptional activation of viral LMP genes and a number of cellular

genes. One of the major target cellular genes activated is c-myc, which in turn activates many other cellular genes and can lead to unchecked cellular growth and division. Three different isoforms exist in EBNA-3, and they inhibit EBNA-2-mediated upregulation of LMP-1 (7). LMP1 acts like a constitutively activated cellular receptor (i.e. CD40). Signaling by CD40 triggers a cascade of events leading to the activation of the important cellular transcription factor, such as nuclear factor kappa B (NF-kB). This is one of the steps that leads to B lymphocyte proliferation during an immune response. LMP-2A also acts like a constitutively activated cellular receptor, the B cell receptor (BCR), which when paired with LMP-1 can drive resting B lymphocytes to proliferate and differentiate into memory B cells. LMP-2A binds to the cellular tyrosine kinases Lyn and Syk, as does an activated BCR. These kinases are activated when the BCR binds to an antigen, leading to a cascade of events that eventually generates plasma cells. LMP-2A has been shown to block signaling through the BCR, perhaps by sequestering Lyn and Syk and therefore preventing their activation; one result is blockage of the activation of EBV virus lytic replication. LMP-2A also shows an anti-apopototic activity (6). Several studies have suggested that LMP-2B can enhance LMP-1 signaling and negatively regulate the function of LMP-2A (2). EBERs may also provide a survival function during latent infection by acting on cellular proteins involved in protein synthesis (e.g. ribosomal protein L22), thereby enhancing cell growth and division. Furthermore, a number of microRNAs (miRNAs) are expressed during EBV latent infection; EBV was the first virus in which miRNA was found. Two independent ebv-miRNAs are known: the ebv-miR-BHRF1 and the EBV-miR-BART families. Viral miRNAs can maintain viral latency, evade host immune response and inhibit lytic cycle reactivation (7). On the basis of the latent gene expression, four different latency patterns are recognized. In type 0 latency, only EBERs are expressed. In type I latency, EBERs and EBNA-1 are expressed. In type II latency, LMP-1, LMP-2A and LMP-2B are additionally expressed. In type III latency, all latent genes are expressed (2). EBV infected naïve B cells in the lymphoid tissue of Waldeyer’s ring in the oropharynx express the full spectrum of latent gene product (latency III, growth program). EBV activates B cells to become proliferating blasts through by the growth program. The naïve infected B cells enter the germinal center (GC) where they proliferate and undergo clonal expansion. The germinal center infected cells exhibit

type II latency (default program). Through the process of the germinal center reaction, these infected GC cells differentiate into memory B cells and enter the peripheral circulation. The EBV-infected memory B cells in periphery expressing only EBERs (latency 0, latency program), thus they are rarely detected by the immune system. However, some of them that express EBNA-1 protein (latency I, latency program) occasionally divide to maintain the long-term reservoir of EBV, which is referred to type III latency (Figure 3) (4).

Figure 3: EBV latent life cycle (Adapted from Fields. Virology. 6th Edition)

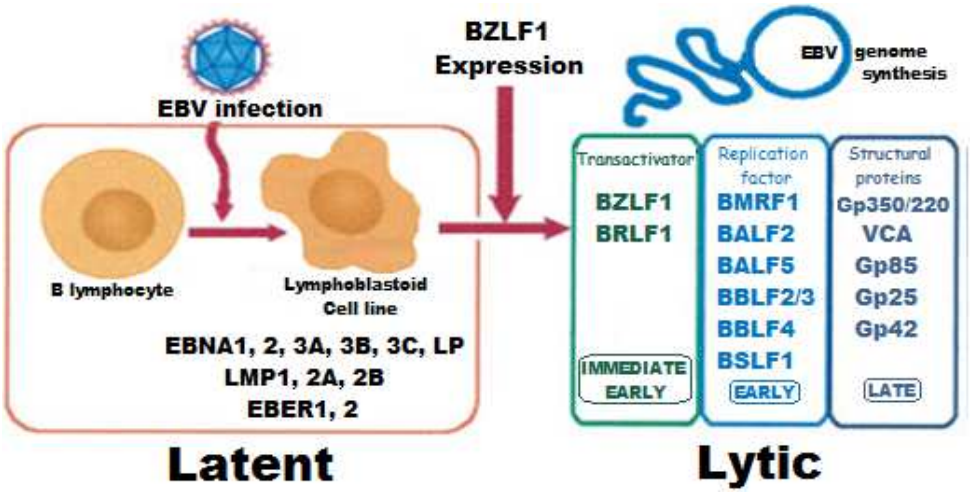


LYTIC EBV INFECTION

In the viral lytic cycle, all the EBV lytic genes (> 80 genes) are expressed coordinately, resulting in amplification of its genome by > 100-fold. Execution of this dynamic cycle produces progeny virus particles, and the cells stop growing and are eventually eliminated by immunity (8). In most asymptomatic carriers of EBV, the virus is periodically replicated and infectious virions can be recovered in oral secretions. Activation of the lytic program appears to occur in latently infected memory B cells recirculating through the lymphoid tissue associated with the oropharyngeal mucosa (9). All herpesviruses encode replication proteins that specifically interact with their origins of lytic DNA replication to provide key functions, including polymerases, helicases, primases, DNA-binding proteins, and associated factors as well as enzymatic activities involved in nucleotide synthesis and phosphorylation (10). Six EBV genes encoding essential lytic functions have been identified: BALF5, BALF2, BMRF1, BBLF4, BSLF1 and BBLF2/3 (11,12). These EBV genes are under the control of two viral

transactivators, BZLF1 and BRLF1, which thus orchestrate transcriptionally the expression of EBV's replication machinery (Figure 4) (10).

Figure 4: Most important viral proteins involved in the latent and lytic phase (adapted from Tsurumi T. et al. Rev. Med. Virol. 2005;15:3-15).



BZLF1 is a key protein for switching from latent to lytic EBV infection; it acts as an oriLyt-binding protein in addition to its function as an immediate-early transactivator and alone is sufficient to activate the EBV lytic cascade (9). The specific interactions that occur among the six viral replication proteins appear to be essential for EBV DNA replication (9).

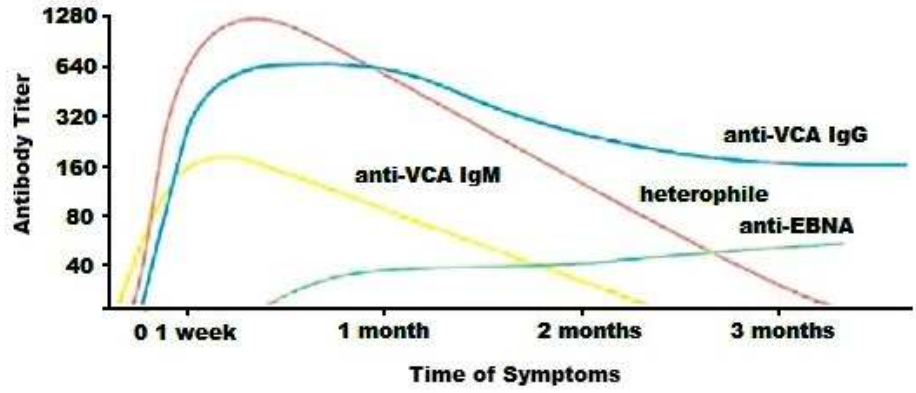
2

IMMUNE RESPONSE TO EBV INFECTION

HUMORAL IMMUNE RESPONSE: ANTIBODY RESPONSE

Acute EBV infection induces polyclonal B cell activation with elevated levels of IgG, IgM, and IgA. Heterophile antibodies are produced, which are not EBV specific. One type of heterophile antibody, used for diagnosis of acute infection, measures the dilution of serum that agglutinates sheep, horse, or cow erythrocytes after absorption with guinea pig kidney. Heterophile antibodies persist for up to 1 year after IM and are often absent in children younger than 5 years old or in the elderly (2). In addition to heterophile antibody, IgM and IgG antibodies to the viral capsid antigen (VCA) are usually present at the onset of symptoms of IM. Later in infection, antibody to EBNA is detected and IgM antibody to VCA disappears (Figure 5). IgG antibody to VCA and EBNA persists for life (2). Antibodies to EBV early antigens (EAs) are classified as either diffuse (EA-D, diffusely in the nucleus and cytoplasm, methanol resistant) or restricted (EA-R, restricted to the cytoplasm, methanol sensitive). EA-D antibodies are often present 3 to 4 weeks after the onset of IM, especially in patients with severe illness, and are frequently detected in patients with nasopharyngeal carcinoma (NPC) or chronic active EBV (CAEBV). EA-R antibodies are often detected in CAEBV or African BL. IgA antibody to EBV is often elevated in patients with NPC, and IgA antibody to EBV EA_S is present in patients with IM (2).

Figure 5: Pattern of antibodies to EBV during acute infection. (From Fields. Virology. 6th Edition).



INNATE CELLULAR IMMUNE RESPONSES: NATURAL KILLER CELLS

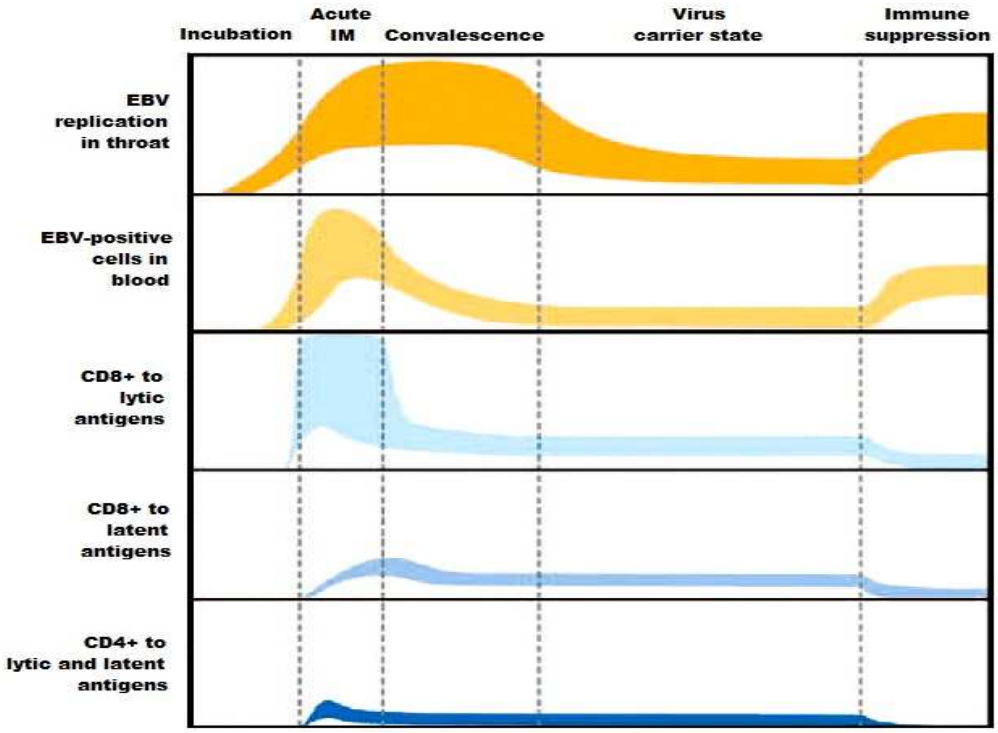
While T cells are thought to constitute the principal effector component of the immune response to EBV, there is mounting evidence that NK cells also play a role (13). Elevated NK cell numbers are associated with lower viral loads in individuals with IM (14). Studies in immunodeficient mice reconstituted with human cells indicate that NK cells are particularly important in controlling lytic EBV infection (15). A recent study suggests that a distinct and stable Interferon (IFN)-γ^{high} NK cell subpopulation found in tonsils of EBV carriers are critical in restricting transformation of B cells (16). NK cells also appear to have a role in controlling chronic viral infection. Individuals with XMEN, a primary immunodeficiency associated with defects in NK cell function, exhibit high levels of EBV and are at increased risk for EBV-related lymphoproliferative disorders and life-threatening IM. Control of chronic EBV infection in these patients has been linked to the NK cell receptor, NKG2D (17) Other rare immunodeficiencies that are NK cell specific have been associated with the development of EBV-related malignancies and life-threatening IM (18, 19).

ADAPTIVE CELLULAR IMMUNE RESPONSES: CD8+ AND CD4+ T CELLS

Although information on the T cell responses to EBV in health and disease is available, CD4+ T cell responses to EBV proteins have been less well studied than CD8+ T cell responses, in particular in IM (2, 13). Initially, CD8+ T cells are directed predominantly against EBV lytic antigens; later in infection cytotoxic T cells (CTLs) are directed to latent antigens. The level of CTLs to EBV immediate-early (IE) antigens (BZLF1, BRLF1) declines rapidly after infection, while the level of CTLs to latency proteins declines much more slowly (Figure 6a). Of the lytic antigens, CD8+ T cells recognize IE proteins (BZLF1, BRLF1) more often than early (E) proteins (BMRF1 and BMLF1), and late (L) proteins least frequently (2). Among the latent proteins, EBNA3A,-3B, -3C, and LMP2-A are the predominant proteins recognized by host CD8+ T cells, although EBNA-1 and LMP-1-specific T cells have also been identified (Figure 6b) (13). Tetramer studies of peripheral blood mononuclear cells (PBMCs) from healthy, seropositive individuals revealed that CD8+ T cells specific for individual immunodominant epitopes of lytic or latent cycle proteins can constitute as much as 2% of the CD8+ T cell subset indicating a significant proportion of the T cell repertoire is devoted to maintaining control of EBV (20). Further, analysis of PBMCs from IM patients revealed that a massive expansion (upwards of 50%) of EBV-specific T cells occurs, with T cells reactive to lytic cycle proteins more abundant than T cells reactive to latent cycle proteins (21). Major histocompatibility complex (MHC) class II tetramers have also been utilized to visualize the CD4+ T cell response to EBV in IM and healthy blood donors. These studies indicate that both lytic and latent proteins are targeted by CD4+ T cells, and while relatively high frequencies of CD4+ T cells can be detected during IM, the overall magnitude of the response is diminished compared to CD8+ T cells (22) (Figure 6a).

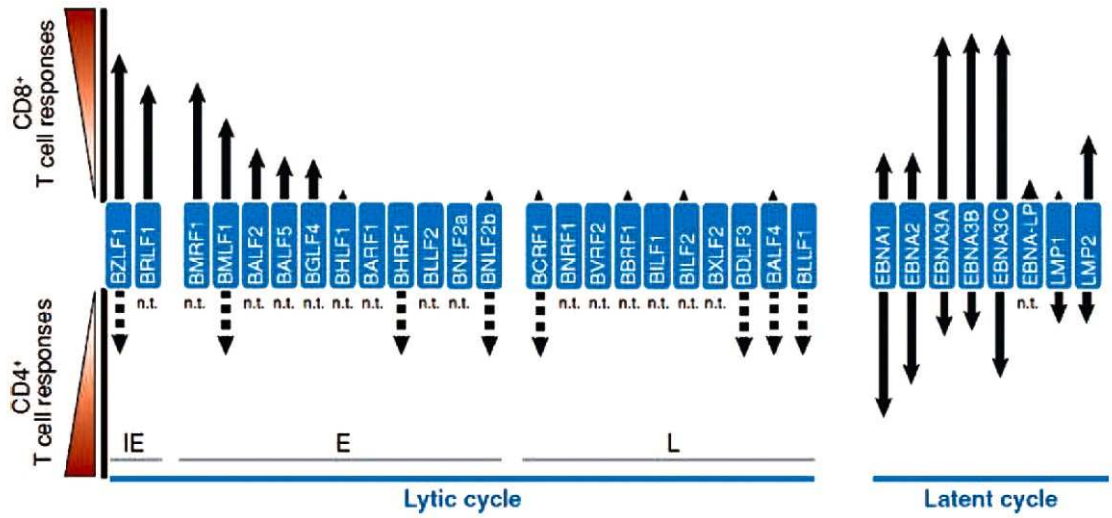
Figure 6: a) Levels of EBV in the throat and blood and cellular immunity during the course of infection; **b)** T cell responses to EBV immediate early (IE), early (E), late (L) and latent proteins. (From Fields. Virology. 6th Edition).

a)



IM: infectious mononucleosis

b)



Dotted arrows indicate that CD4+ T cell responses have been observed to these proteins but that their relative immunodominance is not yet determined; n.t: not tested.

IMMUNE EVASION STRATEGIES

Like other herpes viruses, EBV utilizes a multitude of strategies to evade detection and elimination by the host immune system. Immune evasion strategies of EBV have been recently reviewed and can be broadly classified into three categories, those that modulate: 1) immune cell function, 2) antigen presentation pathways or 3) apoptotic pathways (13). Regarding the modulation of immune cell function, it is recalled the late lytic cycle protein BCRF1, also termed viral IL-10, that can suppress the production of IFN-γ, IL-2 and IL-6 by anti-viral CD4+ T cells (13). A variety of EBV proteins target the processing and presentation of viral antigens thereby promoting immune evasion, e.g. EBNA-1 contains a glycine-alanine repeat that inhibits processing and presentation by HLA class I (13). Furthermore, EBV has evolved several tactics to prevent apoptosis of the infected cell in order to augment viral persistence. A functional bcl-2 homolog encoded by the early lytic cycle protein BHRF1 can inhibit apoptosis induced by a range of stimuli at least in part by binding to the pro-apoptotic protein Bim (13).

3

HEMATOPOIETIC STEM CELL TRANSPLANTATION

The term hematopoietic stem cell transplantation (HSCT) includes a series of procedures in which the patient is treated with either chemotherapy or radiation therapy or both (referred to as the "preparative/conditioning regimen") followed by the infusion of hematopoietic stem cells (HSCs). HSCT can be broadly divided into subtypes based on the donor and the source of HSCs (23). The hematopoietic stem cell donor may be autologous, syngeneic and allogeneic. Specifically:

- In autologous HSCT (auto-HSCT), the HSCs are collected from the patient prior to the administration of high-dose chemotherapy designed to target an underlying malignancy. The reinfusion of the patient's HSCs rescues the patient from long-lasting, usually irreversible, profound pancytopenia. Auto-HSCT is usually not associated with graft-versus-host disease (GvHD). Specifically, an incidence of GvHD post-high dose chemotherapy followed by autologous transplant equal to 4-13% has been recently estimated (24).
- In syngeneic HSCT the HSCs are collected from an identical twin (HLA-identical). These patients do not require post-transplant immunosuppression and do not develop GvHD.
- In allogeneic HSCT (allo-HSCT) the HSCs are collected from a related (which can be HLA-identical, -haploidentical or -mismatched) or an unrelated donor (volunteer or umbilical cord donor) (23). These grafts may be associated with GvHD as well as a potentially beneficial graft-versus-tumor/leukemia (GVL) effect. The period of pancytopenia immediately following HSCT is longer than for autologous HSCT, and immunosuppressive agents are commonly used for prophylaxis against and treatment of GvHD. The combination of these two factors results in a higher rate of complications than after auto- or syngeneic HSCT, especially infectious complications (23).

Regarding the source of the HSCs, it may be: peripheral blood (PB), umbilical cord blood (UCB) and bone marrow (BM). PB stem cells can be collected using hematopoietic growth factors, while BM stem cells are harvested directly from the pelvic bones (23).

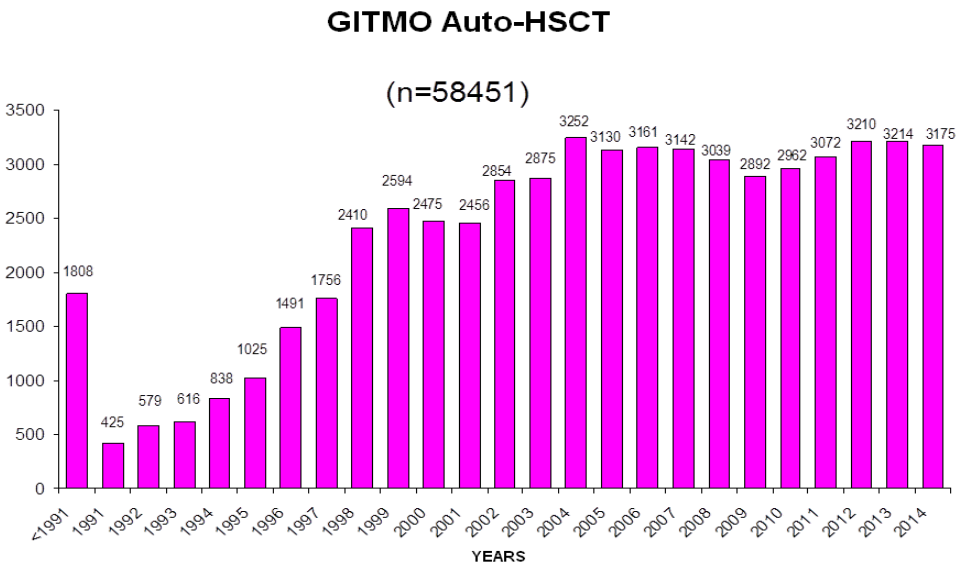
Since the first HSCTs were performed more than 50 years ago, this modality has become a well-established therapeutic option for many hematologic malignancies as well as for BM failure and inborn errors of metabolism. The hematologic malignancies commonly treated with HSCT include acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), myelodysplasia, myeloproliferative disorders, chronic lymphocytic leukemia, chronic myeloid leukemia (CML), multiple myeloma, Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) (25). The nonmalignant disorders include hemoglobinopathies (e.g. sickle cell disease and thalassemia); immune deficiencies (e.g. severe combined immunodeficiency and Wiskott Aldrich syndrome); bone marrow failure syndrome (e.g. aplastic anemia, paroxysmal nocturnal hemoglobulinuria, fanconi anemia); neutrophil disorders (e.g. chronic granulomatous disease); histiocytic disorders (e.g. hemophagocytic lymphohistiocytosis) and lysosomal storage diseases (25).

AUTOLOGOUS TRANSPLANTATION

Auto-HSCT in which the recipient's own HSCs are collected then reinfused after high-dose chemotherapy is used to produce hemopoietic reconstitution after high-dose chemotherapy. This approach allows dose intensification in settings where there is a correlation between dose and tumor response rate and hematopoietic toxicity is a limiting factor for dose intensification. HSCs are harvested and cryopreserved and then reinfused after doses of chemotherapy and radiotherapy that would otherwise be lethal or require a prolonged period of recovery (25). Currently, the most common indications for transplant are myeloma, NHL and HL. It has been shown, in randomized trials or concluded in evidence-based reviews that dose intensification and hemopoietic rescue result in improved long-term disease-free survival (25). However, data from the Center for International Bone Marrow Transplant Research show that the major cause of failure after auto-HSCT is relapse of the primary disease; 69% of cases in transplants carried out in 2011-2012 (26).

Figure 7 shows the number of auto-HSCT performed in Italy in the period of 1991 to December 2014.

Figure 7: Number of auto-HSCT carried out in Italy; period of 1991 - December 2014. (From GITMO, Italian Group of Bone Marrow Transplantation).



ALLOGENEIC TRANSPLANTATION

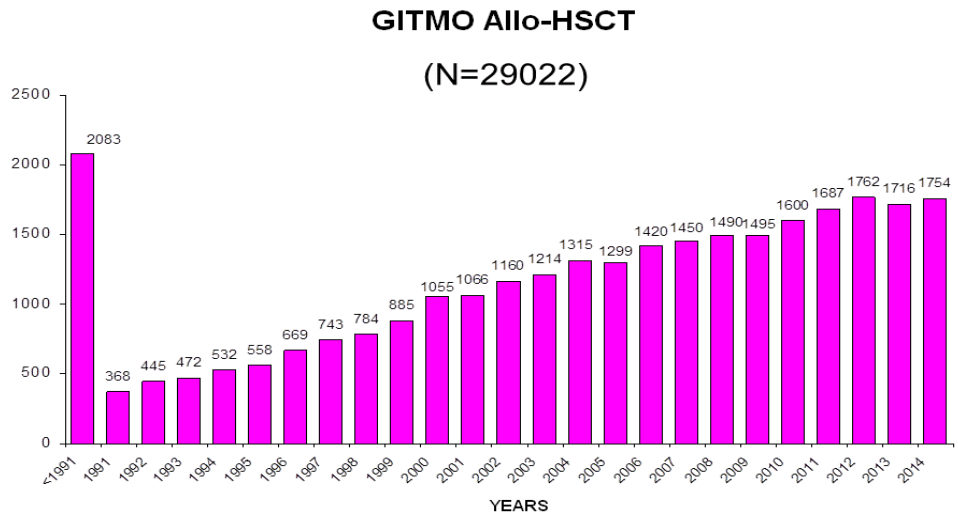
Allo-HSCT uses HSCs collected from someone other than the individual with the disorder. Allo-HSCT is increasingly used to treat a variety of hematologic neoplasms, nonmalignant marrow disorders (acquired and inherited), and inborn errors of metabolism. Eligibility for allo-HSCT varies across countries and institutions and decisions regarding transplant eligibility should be made on a case-by-case basis based on a risk-benefit assessment, the needs and wishes of the patient (23). The selection of a donor is a critical element contributing to the success of a transplant and the major criteria for choosing an allogeneic donor is the degree of histocompatibility between the donor and recipient because the risks of both graft rejection and of GvHD increase with the degree of genetic disparity. The most important determinant of alloreactivity is matching at loci in the MHC which includes HLA, encoded by class I (HLA-A, HLA-B, and HLA-C) and class II (HLA-DR, HLA-DQ, and HLA-DP) genes (25). Other determinants include minor histocompatibility antigens, which are peptides derived from polymorphic proteins that differ between donor and recipient due to single-nucleotide polymorphism (27). NK cells may also contribute to alloreactivity, particularly in the setting of haploidentical transplantation. There is also increasing

evidence that genetic loci outside of the MHC may influence the risk of transplant complications such as infection or regimen-related mortality and several groups are defining genetic variants that might predict these complications (25). The choice of donor for an allo-HSCT depends on several factors including donor choices, the urgency of the transplant and the patient's disease status. The optimal donor is a matched sibling sharing HLA class I and HLA class II alleles, but because each child inherits one set of paternal and one set of maternal HLA antigens, the likelihood of any sibling matching is only 25% (25). When such a donor is not available, a matched unrelated donor (MUD) may be sought. Despite the rapid expansion of donor registries over the past twenty years, availability of MUDs is limited, especially for patients with uncommon HLA genotypes (28). A study evaluated the impact of race and ethnicity on the likelihood of finding an HLA-matched or minimally mismatched donor in the U.S. registry: whites of European descent are the most likely to find an optimal donor (75-97%), whereas blacks from Central or South America are the least likely (16-66%) (29). If an appropriate unrelated donor cannot be found, alternative donors including HLA-mismatched unrelated donors, UCB and related haploidentical donors may be considered (28). Due to the immaturity of the neonatal immune system, a greater degree of HLA mismatching can be allowed for UCBSCs transplantation without excessive GvHD risk. However, the limited number of stem cells present in a UCB unit is a major drawback which is associated with decreased engraftment and delayed immune reconstitution, especially in adult patients, thus limiting the success of UCBSCs transplantation (28). A haploidentical donor can be the patient's parents, siblings, children or other relatives and is matched for half of the MHC alleles of the recipient (one HLA haplotype). Since half of the alleles are mismatched, specific patient treatment and processing of the HSC graft are required in order to avoid severe immunological consequences. In fact, HLA disparities are associated with increased GvHD, graft rejection and poor immune reconstitution (28). It was observed that among patients undergoing unrelated donor BMSC or mobilized PBSC transplant, there is a progressive decrease in post-HSCT survival with each HLA allele mismatch. In particular, each HLA mismatch may reduce the probability of overall survival at five years by approximately 10% (30).

The initial results of transplantation from unrelated donors were inferior to those seen after matched sibling transplantation because of increased incidences of graft rejection and of GvHD caused by the greater genetic disparity (25). Over the past decade, however, results have gradually improved in both single-center and multicenter registry studies, reflecting better donor-recipient matching and advances in GvHD prophylaxis and supportive care. However, one remaining limitation is the time required to identify and screen an unrelated donor, which is approximately equal to 3-6 months. Instead, cord units can be obtained within 1 week of identifying a suitable matched unit. Haploidentical family donors have the greatest genetic disparity but are usually also rapidly available (25).

Figure 8 shows the number of allo-HSCT performed in Italy in the period 1991 to December 2014.

Figure 8: Number of allo-HSCT performed in Italy; period 1991 - December 2014. (From GITMO, Italian Group of Bone Marrow Transplantation).



CONDITIONING / PREPARATIVE REGIMENS

The conditioning regimen has different roles in autologous and allogeneic transplant (25). Patients undergoing an allo-HSCT are prepared with either chemotherapy alone or chemotherapy combined with radiotherapy. The aims are to reduce the tumor burden (when the disease is neoplastic) and suppress the recipient's immune system in order to allow engraftment of stem cells. Exceptions to this rule are infants with combined immune deficiency and patients with severe aplastic anemia (SAA) with an identical twin donor, who may be grafted without conditioning (31). In autologous transplant, the aim of the conditioning regimen is to intensify doses of chemotherapy agents that would be limited by hematopoietic toxicity (25). Conditioning regimens can be defined in three categories: 1) myeloablative (MA) regimens; 2) nonmyeloablative (NMA) regimens and 3) reduced intensity conditioning (RIC) regimens. Assignment to these categories is based on the duration of cytopenia and on the requirement for stem cell support (31). Specifically:

- The term MA refers to the administration of total body irradiation (TBI) and/or alkylating agents, at doses which will not allow autologous hematologic recovery. A MA regimen consists of a combination of agents expected to produce profound pancytopenia and myeloablation within 1-3 weeks from administration; pancytopenia is long lasting, usually irreversible and in most instances fatal, unless hematopoiesis is restored by hemopoietic stem cell infusion. Examples of MA include TBI $\geq$ 5 Gy in a single dose or $\geq$ 8 Gy fractionated or busulfan > 8 mg/kg orally.
- A NMA regimen causes minimal cytopenia and does not require stem cell support. Examples of NMA include fludarabine plus cyclophosphamide with or without antithymocyte immunoglobulin (ATG) or TBI $\leq$ 2 Gy with or without a purine analog.
- RIC regimens are an intermediate category of regimens that do not fit the definition of MA or NMA regimens. RIC regimens differ from NMA since they cause cytopenia, which may be prolonged, and require stem cell support. It is possible that autologous recovery would eventually occur, although pancytopenia may be prolonged to such a degree that is causes significant morbidity and mortality. RIC regimens differ from MA conditioning because the dose of alkylating agents or TBI is reduced by at least 30%. Most often these regimens combine fludarabine with an alkylating agent, melphalan, busulfan, in reduced doses, or fludarabine with reduced dose TBI (31).

There is no standard choice of preparative regimen for HSCT and clinical practice varies across countries and institutions. Usually, a decision regarding preparative regimen is based upon clinical judgement, taking into account details regarding the recipient comorbidities, underlying condition and disease status, donor and graft source (32). All of the preparative regimens have short and long term side effects in addition to myelotoxicity. Common toxicities include mucositis, nausea and vomiting, alopecia, diarrhea, rash, peripheral neuropathy, infertility, interstitial lung disease and sinusoidal obstructive syndrome. Long-term complications following TBI also include asymptomatic alterations in pulmonary function, cataracts, an autoimmune disease (sicca or Sjogren syndrome) and thyroid dysfunction (32).

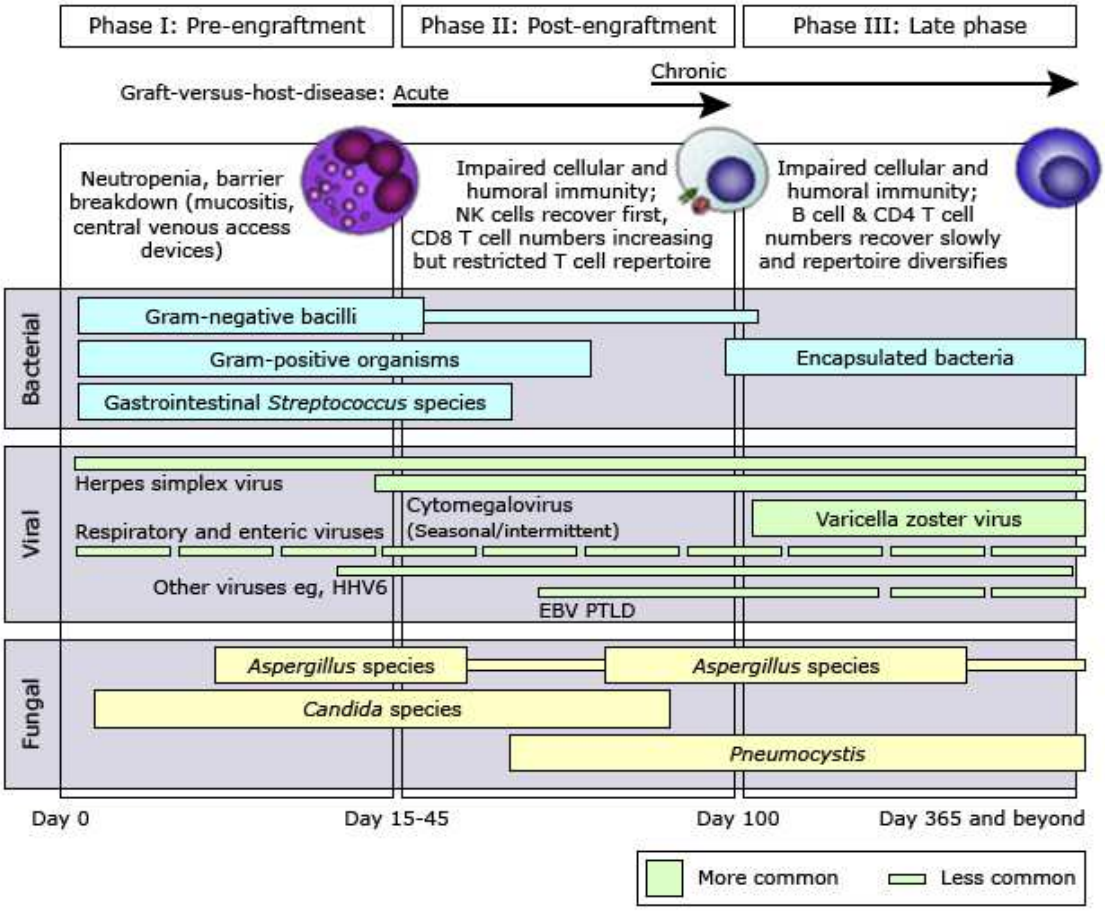
4

EBV INFECTION AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION

Patients who receive transplant are at risk of a number of short- and long- term complications and require long-term follow up (25). Recipients of both allogeneic and autologous transplant have risks of infection during the period of hematopoietic and immune reconstitution as well as early- and long- term complications from toxicities due to the conditioning regimen (e.g. myelodysplasia/secondary leukemia, secondary solid tumors, cardiac and pulmonary toxicity) (23,25). Allograft recipients are also at risk of graft failure and acute and chronic GvHD (occurring < 100 and > 100 days after transplant, respectively) because of the genetic disparity between donor and recipient.

Infections cause up to 7% and to 17% of deaths after auto- and allo-HSCT (26). An international group of experts in infectious diseases, HSCT and Public Health worked together to compile guidelines for preventing infectious complications among HSCT recipients; the document summarizes the current available data in the field (32). The risk of infection, particularly of opportunistic infection, is largely determined by three factors: 1) the patient's net state of immunosuppression (type, degree and duration); 2) the patient’s epidemiologic exposures; and 3) the presence of tissue and/or organ damage (e.g. mucositis, renal failure, lung damage) and/or consequences of the invasive procedures to which the patient is subjected to (33). The type of infections tend to occur at relatively specific times during the post-transplant period and can be divided in three periods, based upon the amount of time elapsed from transplant (Figure 9). Specifically: 1) pre-engraftment phase (I): < 15-45 days after HSCT; 2) early post-engraftment phase (II): 30-100 days after HSCT and 3) late post-engraftment phase (III): > 100 days after HSCT (34).

Figure 9: Timeline and risk factors of opportunistic infection in allo-HSCT recipients. (from Tomblyn M et al. Biol Blood Marrow Transplant 2009;15:1143-1238).



EBV: Epstein-Barr virus; HHV6: human herpesvirus 6; PTLD: post-transplant lymphoproliferative disorders.

During phase I, prolonged neutropenia and breaks in the mucocutaneous barrier result in substantial risk for bacteremia and fungal infections involving *Candida* spp and, as neutropenia continues, *Aspergillus* spp. Additionally, herpes simplex virus reactivation occurs during this phase. During phase II, infections relate primarily to impaired cell-mediated immunity. The scope and impact of this defect is determined by the extent of GvHD and immunosuppressive therapy for it. Herpesviruses are common infectious agents during this period. Other dominant pathogens during this phase include *Pneumocystis jiroveci* and *Aspergillus* spp. During phase III, persons with chronic GvHD and recipients of alternative donor allogeneic transplants remain most at risk for infection. Common pathogens include CMV, varicella-zoster virus and infections with encapsulated bacteria (e.g. *Streptococcus pneumoniae*). The relative risk for these

infections is approximately proportional to the severity of the patient's GvHD during phases II and III. For recipients of NMA grafts, substantial differences may be observed during phase I, but the susceptibility to infections during phases II and III are largely similar and driven primarily by the status of the underlying disease, a history of GvHD and/or the need for ongoing immunosuppression. The risk of disease from community-acquired respiratory viruses is elevated during all 3 phases (34).

Therefore, the risk of infection is primarily determined by the time from transplant and the presence or absence of GvHD. Other factors include donor/host histocompatibility, disease status, graft type, graft contents, conditioning intensity, and neutrophil engraftment. The factors affecting risk of infection are reported in Table 1 (34).

Table 1: Factors affecting risk of infection following HSCT (Adapted from Tomblyn M et al. Biol Blood Marrow Transplant 2009;15:1143-1238).

FACTOR	RISK OF INFECTION
Type of transplant	Higher risk with allogeneic, lower risk with autologous or syngeneic depending on graft manipulation and clinical setting, including previous therapies
Time from transplant	Lower risk with more time elapsed from transplant
Pre-transplant factors	Higher risk with extensive pre-transplant immunosuppressive, prolonged pre-transplant neutropenia or pre-transplant infection
GvHD	Higher risk with grade III-IV acute GvHD or extensive chronic GvHD
HLA match	Higher risk with HLA-mismatched donors, particularly with haploidentical donors
Underline disease status	Higher risk with more advanced disease at the time of transplant
Donor type	Higher risk with unrelated donor than with a fully matching sibling donor
Graft type	Highest risk with cord blood, intermediate risk with bone marrow and lowest risk with mobilized peripheral blood cells. Higher risk with T cell-depleted grafts (depending upon method used)
Immunosuppression after transplant	Higher with immunosuppressive drugs, in particular with corticosteroids, antithymocyte globulin, alemtuzumab
Conditioning intensity	Lower risk in the first 1-3 months post-transplant with low-dose chemo/radiotherapy
Neutrophil engraftment	Higher risk with delayed engraftment/nonengraftment

GvHD: graft-versus-host-disease; HLA: human leukocyte antigen.

As one can observe in Figure 9, the EBV infection occurs commonly from early post-engraftment period onwards. Although fever and mononucleosis can occur in primary EBV infection, the most significant clinical syndrome associated with EBV replication in HSCT recipients is post-transplant lymphoproliferative disorders (PTLD) (34). Since its discovery as the first human tumor virus, EBV has been implicated in the development of a wide range of B-cell lymphoproliferative disorders (35). Specifically, the EBV-associated lymphoproliferative disorders include benign, gray and malignant disorders. In particular, IM lymphadenitis and EBV-positive mucocutaneous ulcer are included in the benign disorders; while CAEBV disease B-cell type and HHV8- and EBV-associated germinotropic lymphoproliferative disorder in the gray ones. Finally, diffuse large B-cell lymphoma of the elderly, diffuse large B-cell lymphoma associated with chronic inflammation, plasmablastic lymphoma, primary effusion lymphoma, lymphomatoid granulomatosis, BL, classical HL and PTLD are included in the malignant disorders (7). Furthermore, EBV has been associated with other human cancers including NPC, gastric carcinoma and T-cell lymphoproliferative disorders such as peripheral T-cell lymphomas, angioimmunoblastic T-cell lymphoma, extranodal nasal type NK/T-cell lymphoma and other rare histotypes (2,35).

In Table 2, particular EBV-driven lymphoproliferative disorders with specific latency patterns are showed (7).

Table 2: Patterns of EBV latent gene expression in EBV-driven lymphoproliferative disorders. (from Ok CY et al. Experimental & Molecular Medicine 2015;47, e132).

Latency	Gene products	Disease
I	EBER, EBNA-1	BL, PBL, PEL
II	EBER, EBNA-1, LMP-1, LMP-2A, LMP-2B	CHL, EBV^+ DLBCL of the elderly, PBL (subset), PTLD (subset)
III	EBER, EBNA-1, LMP-1, LMP-2A, LMP-2B, EBNA-2, EBNA-3A, -3B, -3C	IM, DLBCL with CI, LyG, PTLD, EBV^+ DLBCL of the elderly (subset)

EBER: Epstein-Barr virus encoded small RNA; EBNA: Epstein-Barr virus nuclear antigen; LMP: latent membrane protein; BL: Burkitt lymphoma; PBL: plasmablastic lymphoma; PEL: primary effusion lymphoma; CHL: classical Hodgkin lymphoma; DLBCL: diffuse large B-cell lymphoma; LyG, lymphomatoid granulomatosis; PTLD: post-transplant lymphoproliferative disorder; IM: infectious mononucleosis; DLBCL with CI: diffuse large B-cell lymphoma associated with chronic inflammation.

5

EBV-RELATED PTLD

PTLD is increasingly recognized as an important cause of morbidity and mortality in both solid organ transplant and HSCT recipients. In solid organ transplant patients, the cells giving rise to PTLD are usually of recipient origin, suggesting a role of endogenous EBV reactivation in these patients. Conversely, in allo-HSCT, PTLD is donor-derived as a consequence of the complete eradication of the host lymphoid system by conditioning regimens (36). A revised classification was published in 2008 by the World Health Organization (WHO) and uses morphologic, immunophenotypic, genetic, and clinical features to define four main categories of PTLD such as early lesions, polymorphic, monomorphic and CHL-type PTLD (37). All types are associated with EBV (38). Fever, generalized lymphadenopathy, respiratory compromise and rising liver transaminase levels are typical and have usually been associated with a rapidly progressive multi-organ failure and death. Lesions are nodal and extra-nodal, frequently involving Waldeyer's ring, the gastrointestinal tract, the liver, and the central nervous system. PTLD that arise later after transplantation (>1 year, late EBV-PTLD) are more commonly localized and often have an indolent course. On the contrary, patients with EBV infection usually asymptomatic initially (39). Risk factors for development of PTLD include type of donor, T-cell depletion, use of ATG, splenectomy and EBV serology mismatch between donor and recipient (40). In a recent large, multi-center and retrospective analysis, the overall EBV-related PTLD frequency observed was of 3.22%, ranging from 1.16% for matched-family donor to 11.24% in mismatched unrelated donor recipients (41). EBV-PTLD incidence varies markedly with the time after transplantation, with particularly high rates occurring during the first 5 months, followed by a steep decline in incidence between 6 and 12 months post-transplant (early PTLD < 1 year). A significantly increased risk of PTLD continues among longer term survivors, although the rate is greatly diminished. The high incidence of PTLD during the first few months after transplant is consistent with clinical investigations of the temporal pattern of immune reconstitution in HSCT recipients (42). In fact, the incidence decreases due to the increase in EBV-specific T cell (43). Late PTLD after HSCT (> 1 year) differ in their risk factors, pathology and

EBV association. These tumors are sometimes of T cell origin rather than B cell origin, and at times are not EBV associated (39). In fact, the EBV genome is found in more than 90% of B-cell PTLD occurring during the first year after transplantation (in the early phase), while up to 45% of late onset PTLD may be EBV negative (36). The diagnosis of EBV-PLTD requires biopsy with in situ hybridization or immunochemistry to define viral association. EBV-encoded RNA in situ hybridization is the most sensitive tool for detecting virus in tumor. LMP-1 staining is also available in most pathology laboratories but is negative in the subset of tumors that do not express the viral antigen. Immunohistochemistry for EBNA-1 could also be broadly applicable (39). One year post-transplant, chronic GvHD, an established correlate of immune dysregulation in long-term survivors, has been identified as a strong risk factor for late-onset PTLD. Thus, it is likely that risk factors associated with altered immunity and T-cell regulatory mechanisms might be predictors of both early and late onset PTLD (44).

MECHANISMS FOR EBV-DRIVEN PTLD

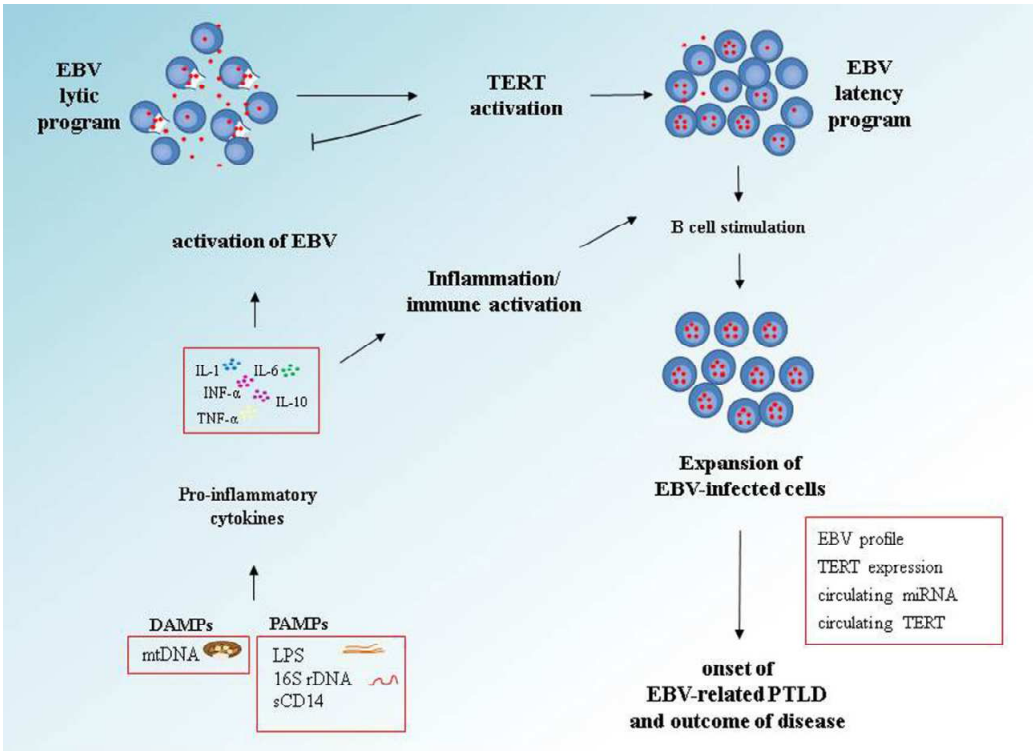
EBV is associated with both B cell and epithelial cell malignancies. As previously described, EBV has both latent and lytic programs in its life-cycle. Since lytic EBV replication triggers the death of infected cells, tumors require the expression of latent programs. Among the EBV latent proteins, LMP-1 is the main oncogenic one and is essential for EBV-driven tumorigenesis (45). As previously reported, this protein activates several downstream signaling pathways, which contribute to the expression of anti-apoptotic proteins and cytokines by acting like CD40. Like most cancers, EBV-associated malignancies require induction of telomerase activity. Telomerase, a ribonucleoprotein complex containing an internal RNA template and a catalytic protein with telomere reverse transcriptase (TERT) activity, extends the telomeres at the ends of eukaryotic chromosomes, thus preventing cell senescence and apoptosis. The internal RNA template is constitutively present in normal and tumor cells, while TERT is the rate-limiting component of the telomerase complex and its expression correlates with telomerase activity. TERT activity is repressed in somatic tissues, but both TERT expression and telomerase activity are elevated in most human tumors. In EBV-infected primary B lymphocytes, activation of TERT occurs concomitantly with the induction of latent EBV proteins and down-regulation of EBV lytic gene expression. LMP-1 is the

main driver of EBV-induced immortalization since it activates TERT. In turn, TERT expression negatively affects the expression of BZLF-1, the master regulator of the viral lytic cycle, thereby favoring the induction and maintenance of EBV latency, a prerequisite for EBV-driven transformation. In contrast, TERT silencing induces the expression of BZLF-1, EA-D and gp350 EBV lytic proteins and triggers a complete lytic cycle. Although latency programs predominate in EBV-driven tumors, some recent data have suggested that the uncontrolled viral lytic cycle has some pathogenic importance, at least in the early phase of transformation. In fact, lymphoblastoid B cell lines obtained with lytic-defective EBV strains have been shown to be less effective in inducing EBV-positive lymphoproliferations in severe combined immunodeficiency mice, an effect due to the lower production of the B-cell growth promoting factors IL-6, cIL-10 and vIL-10, dependent on BZLF-1, the main EBV lytic transactivator. Moreover, several EBV lytic proteins have also been shown to favor immune evasion by inhibiting the synthesis of IFN-γ and suppressing CD8 cytotoxic T cells and to contribute to tumorigenesis by promoting angiogenesis. Besides immune depression, persistent immune activation/chronic inflammation may also play a key role in PTLD development. Studies have identified parallels between cancer and infectious disease. Indeed, in cancer as in infectious diseases, the release of microbial pathogen-associated molecular patterns (PAMPs - such as lipopolysaccharide, 16S ribosomal DNA and CpG DNA) and endogenous damage-associated molecular patterns (DAMPs - such as mitochondrial DNA) by engaging the extra- or intracellular domain of Toll-like receptors may initiate a complex signal transduction cascade which ultimately leads to the release of pro-inflammatory cytokines, such as IL-6, IL-10, IFN-α and tumor necrosis factor, causing chronic inflammation. This chronic stimulation may activate EBV replication and/or contribute to the polyclonal expansion of EBV positive cells. Chronic B-cell hyperactivation is driven by overproduction of B-cell stimulatory cytokines, such as IL-6, IL-10, IFN-α and tumor necrosis factor (45).

In Figure 10 a schematic depict of the pathogenetic mechanisms of early onset EBV-PTLD is reported (45). Briefly, PAMPs and DAMPs, through Toll-like receptors, promote the release of pro-inflammatory cytokines, which in turn activate EBV lytic cycle. At early stage of disease, EBV lytic replication leads to increased number of infected cells. Moreover, B-cell activation, due to persistent inflammation/immune

activation, may also favor expansion of EBV-infected B cells, a crucial step for PTLD development. Along with infection, the oncogenic LMP-1 viral protein transcriptionally activates TERT which in turn blocks lytic viral replication. EBV-infected B cells expressing LMP-1 and cellular TERT protein are prone to transformation (45).

Figure 10: Pathogenetic mechanisms of early onset EBV-PTLD. (from Petrara MR et al. Cancer Letters 2015; 369:37-44).



DAMPs: damage-associated molecular patterns; PAMPs: pathogen-associated molecular patterns; IL: interleukin; INF: interferon; TNF: tumor necrosis factor; TERT: telomere reverse transcriptase; miRNA: microRNA

STRATEGIES FOR TREATMENT OF EBV-DRIVEN PTLD

There is no consensus treatment model for the optimal management of PTLD due to its clinico-pathologic heterogeneity. Strategies to prevent EBV-associated PTLD usually aim at partially recovering immune surveillance and involve reduction of immune suppression to allow restoration of specific immunity and control of EBV-infected proliferating cells (45). Strategies for treatment of EBV-driven PTLD include:

- Reduction of immunosuppressive therapy, if possible. It appears to be effective in 23-50% of cases, although the difference in response seems to be related to the degree

of reduction of immunosuppression or to the time of disease onset. Patients with early-onset disease have better outcomes with reduced immunosuppression than those with late-onset PTLD (46).

- Anti-CD20 monoclonal antibody rituximab 375 mg/m^2, weekly doses - the number of doses are assessed locally on the basis of changes in EBV DNA load. It is the first line of treatment for patients who do not respond to reduction or discontinuation of immunosuppression (45). In several studies, rituximab administered for treatment purposes yielded a 40-68% response rate (47-49). In the preemptive setting, it has been demonstrated that the anti-CD20 antibody can prevent EBV-associated PTLD in about 90% of cases (50).
- EBV-specific cytotoxic T cells infusion, if available. The most brilliant results were obtained in the pre-emptive setting, whereas the clinical benefit in patients with overt PTLD is still unsatisfactory in a proportion of cases (45).
- Antiviral agents. The limited data available on antiviral agents in patients with EBV reactivation and PTLD following allo-HSCT does not support their use. The main cause of PTLD is the proliferation of the latently infected EBV-positive B cells - and not lytic replication - which is why antiviral pharmacotherapy is not expected to be effective in this setting (39). At this regard, there is increasing interest in developing strategies potentially able to efficiently reactivate EBV lytic gene expression in latently infected tumor cells to treat overt EBV-associated malignancies, as lytic infection promotes the death of EBV- positive tumor cells both *in vitro* and *in vivo*. Combination of antivirals with lytic cycle inducers is emerging as a highly promising strategy in treating EBV-driven tumors. In view of the role of TERT in regulating the switch from latent to lytic infection, a strategy aimed at inhibiting TERT in combination with antiviral drugs appears particularly attractive (45).

6

MANAGEMENT OF EBV INFECTION: STATE OF THE ART

Prompt diagnosis is surely necessary due to the rapid and often disseminated nature of PTLD in HSCT recipients (51). Several studies have clearly shown that the development of PTLD is in most cases, both after solid organ and stem cell transplant, accompanied by a significant increase in EBV DNA level in peripheral blood (52-57). Increasing EBV DNA levels rather than a stable-elevated EBV DNA load seems to be a more reliable marker of PTLD (58). Therefore, serial monitoring is important for identification of patients at risk of disease, distinguishing patients with a stable-elevated EBV DNA load from those with increasing EBV DNA levels (39). While it is accepted that EBV DNA levels in the blood of patients with EBV-related PTLD are significantly higher than those in transplant recipients without PTLD (52-57), to date the choice of optimal clinical specimen - whole blood (WB), plasma or PBMCs - for the monitoring of EBV infection in transplanted patients is a matter of debate (59-62). Different assays use WB, plasma or PBMCs, all requiring different interpretation. The choice of unit of measurement also remains controversial (63). In general, assays using PBMCs are able to predict EBV disease at the earliest stage. Elevated EBV DNA in PBMCs may reflect both EBV in normal B cells (a population that may be expanded in immunosuppressed patients) and EBV in transformed cells. EBV DNA in plasma might reflect virus shedding, which occurs intermittently from lytically infected B cells, as well as virus released from necrotic cells. EBV load in WB reflects the combination of all sources (63). Several studies comparing EBV DNA determination in PBMCs, WB, and plasma using real-time PCR assays showed higher analytical sensitivity for PBMCs and WB (38,59,64-66). In addition, there is no international consensus on what is the EBV DNA threshold level predictive for developing PTLD and then for the need of pre-emptive therapy. In fact, various data about this issue have been reported and have been related to local experiences (50, 67-72).

Clinically, the presence of high or increasing levels of EBV DNA in peripheral blood has been used as a parameter to modulate the immunosuppressive therapy and to start pre-emptive or antitumor therapy (73). Nevertheless, the detection of increased EBV load alone is not always predictive of impending PTLD (45). In fact, studies have

demonstrated that the outcome of virus infections depends on the efficacy of specific T cell responses (74). Numerous studies have focused on immune monitoring of human cytomegalovirus (CMV) in transplant recipients, while monitoring of EBV-specific T cell responses and their association with EBV-related complications after transplant remain largely unexplored (74). Furthermore, it should be note that in recent years there has been a growing interest in the development of immune monitoring approaches that may allow the identification of infectious risk in transplant recipients and, eventually, the modulation of immunosuppressive strategies by using non pathogen-specific assays (i.e. quantification of cytokines and chemokines, enumeration of T-cell subsets and ImmuKnow assay) (74).

At this regard, in our Centre at St. Orsola-Malpighi University Hospital, before the launch of this project involving HSCT recipients, we studied the potential additive value of immunological assays, both pathogen-specific and non pathogen-specific, in the post-transplant setting of solid organ transplant recipients. Specifically, we assessed *i)* the utility of measuring CMV-specific cell-mediated immunity (CMI) by Enzyme-linked ImmunoSPOT assay in bowel/multivisceral transplant recipients to provide additional information on the risk of infection and development of CMV disease (75); *ii)* the clinical benefits of adjusting immunosuppressive therapy in liver transplant recipients based on a non-pathogen- specific immune function assay results such as ImmuKnow™ assay (Interventional group) compared to a group managed with the standard clinical practice (Control group). Among the primary outcomes, the incidence of infection between the two groups was compared in order to establish if ImmuKnow™ assay provides a useful biomarker which enables optimizing immunosuppression to improve patient outcomes by preventing infections (76) (See the two papers in Part VII - Annexes). Finally, we performed the monitoring of CMV-specific CD8+ T cell responses using the QuantiFERON®-CMV assay in heart transplant recipients. Specifically, the pattern of CMV-specific CMI reconstitution after heart transplant was evaluated and the clinical utility of the assay for the management of post-transplant CMV infection was assessed ("Monitoring of CMV-specific cell-mediated immunity in heart transplant recipients: clinical utility of the QuantiFERON®-CMV assay for the management of CMV infection"; submitted manuscript - J Clin Virol).

Part II

AIM OF THE PhD PROJECT

To date, the diagnosis and monitoring of EBV infection are based on direct evaluation of viral burden by quantitative molecular methods such as real-time PCR assay. However, these tests do not provide information regarding the host immunological response against the EBV replication, thus often failing in the prediction of infection progression to overt disease.

The general objective of the PhD research was to assess the utility of measuring EBV blood replication combined with the specific viral cellular immunity in allo-HSCT recipients to monitor the risk of development of EBV-PTLD. Moreover, the kinetics of EBV DNA in two different blood compartments - WB and PBMCs - were studied.

In particular the PhD project, that involved both adult and paediatric allo-HSCT recipients, was focused on the following specific topics:

1. The incidence of EBV infection and the potential risk factors for the development of active EBV infection.

2. Suitability of WB as clinical specimen to monitor the risk of developing EBV-PTLD.

3. Combined virological-immunological monitoring of EBV infection as a possible predictive marker of EBV-related complications.

4. The management of EBV infection and EBV-PTLD prevention.

Part III

STUDY DESIGN

Consecutive adult and paediatric patients who underwent allo-HSCT during the period 2014 - 2015, respectively at the Institute of Hematology and Medical Oncology "L. and A. Seràgnoli" and at the Operative Unit of Paediatrics - Oncology, Haematology, and Stem Cell Transplantation Program - of the St. Orsola-Malpighi University Hospital of Bologna were enrolled. The study was approved by the St. Orsola-Malpighi University Hospital Research Ethics Committee (VIRO-13 Study; 41/2013/U/Tess - see the related documents in Part VII - Annexes) and all patients provided informed consent. As a starting point, the EBV serostatus in paired Donor/Recipients (D/R) was determined to identify EBV risk groups: D-/R- and D+/R- (EBV primary infection); D-/R+ and D+/R+ (EBV reactivation/reinfection). For all patients, routine virological monitoring of EBV infection was performed on WB samples by a quantitative real-time PCR. Virological monitoring was performed every week during the first 100 days post-transplant and every 2 weeks until 180 days post-transplant. Afterwards, blood samples were processed if clinically indicated. Since EBV DNA is typically harbored within latently infected B-lymphocytes, very low EBV DNA levels may be present in WB samples in the setting of latent infection. Therefore, we established that an active EBV infection was defined as the detection of EBV DNA greater than 500 copies/mL WB in at least two consecutive samples. After the first EBV DNA positivity in WB samples $\geq$ 500 copies/mL, EBV DNA load in PBMCs will be also prospectively monitored starting from the next visit. Furthermore, in the case of EBV DNAemia, monitoring was performed weekly for at least 1 month. Immunological monitoring of EBV infection was carried out using Enzyme-linked ImmunoSPOT (EliSpot) assay that enumerates Interferon-gamma-secreting EBV-specific T cells (both CD4+ and CD8+ cells), at a single cell level, upon *in vitro* stimulation with latent and lytic viral antigens. EliSpot assay was performed on patients' blood samples at days 60, 100, 180 and 360 after allo-HSCT. Moreover, for the patients at higher risk of developing EBV-PTLD, i.e. patients who showed high viral load, EliSpot assay was performed during the next visit. Specifically, based on previous studies (77,78) and on the GITMO guidelines for the diagnosis and treatment of EBV-PTLD in HSCT recipients, any values of EBV DNA $\geq$ 10,000 copies/mL WB and $\geq$ 1,000 copies/1x10^5 PBMCs were defined as high viral load. In addition, all patients were monitored for CMV infection during the post-

transplantation period. CMV real-time PCR was performed weekly during the first three months and then monthly until the sixth month. Afterwards, blood samples were processed if clinically indicated.

Patients underwent virological-immunological and clinical monitoring for at least one year post-transplant.

Part IV

RESULTS

1

THE INCIDENCE OF EBV INFECTION AND THE POTENTIAL RISK FACTORS FOR ITS DEVELOPMENT

INCIDENCE OF EBV INFECTION

The overall incidence of post-transplant active EBV infection in adult and paediatric patients was equal to 45.5% and 61.1%, respectively. Specifically, in the adult study population the incidence of post-transplant primary EBV infection and EBV reactivation/reinfection was equal to 3% and 42.4%, respectively. No paediatric patients were EBV-seronegative at the time of transplant.

ADULT STUDY POPULATION

Thirty-seven adult allo-HSCT recipients were enrolled. Four patients (10.8%) were excluded from the study because they died in the immediate early post-transplant period (median time 7 days post-transplant; range, 5 - 42). For all the four patients the source of stem cells was CB and the donor type was a MUD; the causes of death were relapse of the original disease (n = 2; LAM) and respiratory failure (n = 1). Among the 33 evaluable patients, 21 were males and 12 females with a mean age of 40 years (range, 18 - 59). The study population's transplant characteristics are reported in Tables 3 (patients with active EBV infection; n = 15) and 4 (patients not actively EBV infected; n = 18).

Table 3: Characteristics of 15 adult patients who developed an active post-transplant EBV infection and results of virological monitoring. Patients were disposed in the Table on the basis of the viral load detected during the follow-up of EBV infection: from highest to lowest EBV DNAemia values.

Pt	Age years	Sex	Primary disease	Donor type	Graft origin	EBV serology	Conditioning regimen	T-cell depletion	GvHD prophylaxis	EBV DNAemia whole blood copies/mL
9	40	M	ALL	MUD	BM	D?/ R+	TBI-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 522 – 119,039
21	41	F	AML	MUD	CB	D?/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MMF	Min-max 1837 – 74,374
7	34	F	HL	MUD	PBSC	D+/ R+	RIC regimen	*in vivo*	CyA + MTX	min-max 4650 – 55,769
17	35	F	AML	RELATED Sibling	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 1433 – 15,820
18	33	M	AML	MUD	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 517 – 10,966
6	49	F	AML	RELATED Sibling	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 623 – 10,521
16	57	M	CML	MUD	PBSC	D?/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 564 – 7,279
5	59	M	AML	MUD	PBSC	D+/ R+	RIC regimen	*in vivo*	CyA + MTX	min-max 678 – 3,386
2	18	M	ALL	MUD	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min- max 510 – 3,147
27	22	M	CML	MUD	PBSC	D-/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 537 – 3,035
12	53	F	AML	MUD	BM	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	Only MTX	min-max 603 – 2,657
19	55	F	ALL	MUD	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 686 – 1,769
34	43	F	ALCL	RELATED Sibling	PBSC	D-/R+	RIC regimen	*in vivo*	CyA + MTX	min-max 631 – 1,703
23	35	M	AML	MUD	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 720 – 1,611
4	20	M	ALL	RELATED Sibling	PBSC	D+/ R-	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 514 – 1,038

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; HL, Hodgkin lymphoma; CML, chronic myeloid leukemia; ALCL, anaplastic large cell lymphoma; Pt, patient; M, male; F, female; MUD, matched unrelated donor; PBSC, peripheral blood stem cell; BM, bone marrow; CB, cord blood; D, donor; R, recipient; TBI, total body irradiation; RIC, reduced-intensity conditioning; CyA, cyclosporine A; MTX, methotrexate; MMF, mycophenolate mofetil; GvHD, graft-versus-host disease.

Table 4: Characteristics of 18 adult patients who resulted negative for EBV DNA over the entire follow-up.

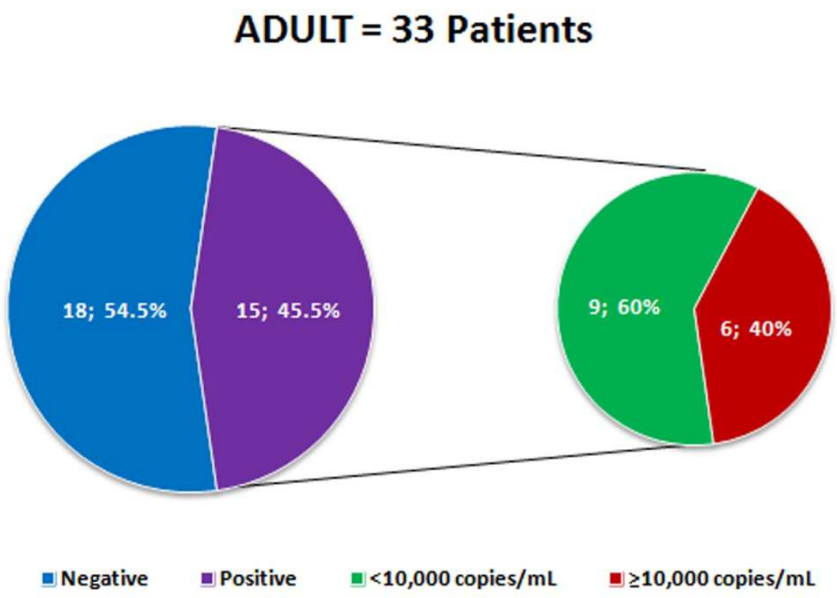
Pt	Age years	Sex	Primary disease	Donor type	Graft origin	EBV serology	Conditioning regimen	T-cell depletion	GvHD prophylaxis	EBV DNAemia whole blood copies/mL
1	51	M	AML	MUD	PBSC	D?/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
3	24	M	AML	MUD	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
8	43	M	AML	MUD	BM	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
10	42	M	AML	MUD	BM	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
11	45	F	AML	MUD	CB	D?/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MMF	Negative
14	25	F	AML	MUD	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	Only CyA	Negative
20	34	M	MM	MUD	CB	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
22	50	M	DLBCL	MUD	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
24	37	M	ALL	MUD	PBSC	D-/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
25	22	M	ALL	RELATED Sibling	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
26	21	M	ALL	MUD	BM	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
28	39	F	MS	RELATED Sibling	BM	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
29	38	M	AML	MUD	BM	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
30	43	F	CML	MUD	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
31	51	M	MDS	MUD	BM	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
32	56	M	AML	MUD	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
33	46	M	MM	RELATED Sibling	PBSC	D+/ R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
35	47	F	MDS	MUD	PBSC	D-/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative

AML, acute myeloid leukemia; MM, multiple myeloma; DLBLC, diffuse large B-cell lymphoma; ALL, acute lymphoblastic leukemia; MS, myeloid sarcoma; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; Pt, patient; M, male; F, female; MUD, matched unrelated donor; PBSC, peripheral blood stem cell; BM, bone marrow; CB, cord blood; D, donor; R, recipient; RIC, reduced-intensity conditioning; CyA, cyclosporine A; MTX, methotrexate; MMF, mycophenolate mofetil; GvHD, graft-versus-host disease.

Most patients (48.5%) underwent transplant for AML. The donor type was a MUD for 26 allo-HSCT (78.8%) and a related donor type for 7 (21.2%) patients; in all transplants the donor was HLA-identical sibling. The source of stem cells was PB in 66.7% of transplants (22/33 patients). At the time of transplant, the EBV serostatus of D/R were: D+/R+ in 23/33 patients (69.7%), D+/R- in 1/33 patients (3%) and D-/R+ in 4/33 patients (12.1%). The serology of the donor was not available in 5 cases (15.2%); all the respective recipients were EBV-seropositive. The conditioning regimen was of reduced intensity for 3 patients and MA for 30 patients. All 33 patients underwent *in vivo* T-cell depletion to reduce rejection and GvHD. On the basis of the patient-related and transplant related characteristics, ATG rabbit (GRAFELON formerly ATG-Fresenius; Neovii Biotech GmbH, Graefelfing, Germany), in a total dose ranging from 15 - 30 mg/kg, was administered over 5 days, from day -6 to -2. In most cases, GvHD prophylaxis consisted of an association of cyclosporine A and methotrexate (n = 29).

The 33 adult allo-HSCT recipients were monitored for EBV infection for a median time of 12 months post-transplant (range, 5 - 17). For 18 out of 33 patients (54.5%), WB samples tested negative for EBV DNA over the entire follow-up. Fifteen patients (45.5%) developed an active post-transplant EBV infection: 11 patients who underwent allo-HSCT from MUD (42.3%, 11/26 patients) and 4 patients transplanted from related donor (57.1%, 4/7 patients). One out of 15 patients (6.7%) developed primary EBV infection and 14/15 (93.3%) patients developed EBV reactivation/reinfection. Among the 15 patients with an active post-transplant EBV infection, 9 patients (60%) showed low values of EBV DNAemia (< 10,000 copies/mL WB), whereas 6 patients (40%) showed values of EBV DNAemia equal or greater than 10,000 copies/mL WB. Two out of 15 (13.3%) actively EBV infected patients developed EBV-related complications such as mononucleosis-like syndrome - fever and pharyngitis - (patient number 21) and EBV-PTLD (patient number 9). The results of the virological follow-up are reported in Figure 11.

Figure 11: Results of the virological follow-up - Adult patients.



The median peak of viral load in patients with low and high values of EBV DNAemia was 2,657 copies/mL (range, 1,038 - 7,279 copies/mL) and 35,794 copies/mL (range, 10,521 - 119,039 copies/mL), respectively. The median time at which EBV DNAemia was first detected was 52 days (range, 20 - 474) post-transplant. The results obtained by virological monitoring, along with the range of EBV DNAemia values detected per patient, are reported in Table 3.

PAEDIATRIC STUDY POPULATION

Twenty-one paediatric allo-HSCT recipients were enrolled. Among the 21 patients, 3 patients (14.3%) died approximately two months post-transplant (median time 66 days post-transplant; range, 57-67). Two of them received a HSCT from a related donor and died of multi-organ failure - BM and combined BM-CB were the sources of stem cell; the remaining patient underwent allo-HSCT from MUD, BM was the source of stem cells and the cause of death was relapse of the original disease (AML). Then, the paediatric study population included 18 evaluable patients. The mean age was 9 years (range, 9 months - 20 years) and the male/female ratio was of 13/5. Study population's transplant characteristics are reported in Tables 5 (patients with active EBV infection; n = 11) and 6 (patients not actively EBV infected; n = 7).

Table 5: Characteristics of 11 paediatric patients who developed an active post-transplant EBV infection and results of virological monitoring.

Patients were disposed in the Table on the basis of the viral load detected during the follow-up of EBV infection: from highest to lowest EBV DNAemia values.

Pt	Age years	Sex	Primary disease	Donor type	Graft origin	EBV serology	Conditioning regimen	T-cell depletion	GvHD prophylaxis	EBV DNAemia whole blood copies/mL
21	15	M	SAA	RELATED Sibling	PBSC	D+/R+	RIC regimen	*in vivo*	CyA + MTX	min- max 872 – 2,011,688
17	4	M	ALL	MUD	BM	D+/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min- max 631 – 683,294
4	10	M	ALL	MUD	PBSC	D+/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 1120 – 89,724
20	6	M	β-TM	MUD	BM	D?/R+	RIC regimen	*in vivo*	CyA + MTX	min- max 2813 – 45,548
18	1	M	AML	RELATED Sibling	BM	D+/R+	Busulfan-based myeloablative regimen	NO	Only CyA	min-max 1,289 – 15,312
8	8	M	ALL	RELATED Sibling	BM	D-/R+	Treosulfan-based myeloablative regimen	NO	Only CyA	min- max 522 – 14,787
3	14	M	AML	MUD	BM	D+/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 820 – 12,395
2	8	M	ALL	MUD	BM	D?/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 560 – 10,763
1	8	M	ALL	MUD	BM	D+/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	min-max 513 – 3,412
19	9	M	β-TM	RELATED Sibling	BM	D+/R+	RIC regimen	*in vivo*	Only CyA	min- max 546 – 2,727
7	13	F	AML	RELATED Sibling	BM	D?/R+	Busulfan-based myeloablative regimen	NO	Only CyA	min-max 509 – 1,033

SAA, severe aplastic anemia; ALL, acute lymphoblastic leukemia; β-TM, beta-thalassemia major; AML, acute myeloid leukemia; Pt, patient; M, male; F, female; MUD, matched unrelated donor; PBSC, peripheral blood stem cell; BM, bone marrow; D, donor; R, recipient; RIC, reduced-intensity conditioning; CyA, cyclosporine A; MTX, methotrexate; GvHD, graft-versus-host disease.

Table 6: Characteristics of 7 paediatric patients who resulted negative for EBV DNA over the entire follow-up.

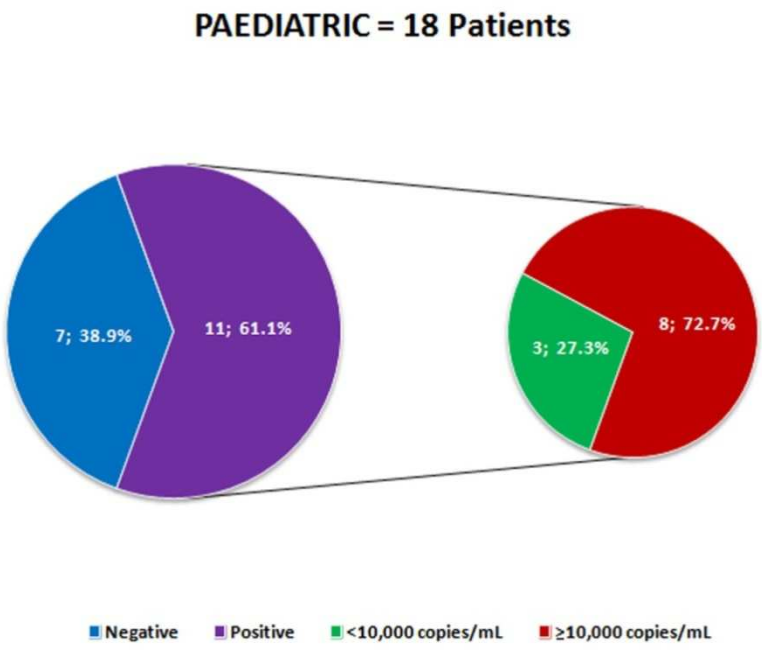
Pt	Age years	Sex	Primary disease	Donor type	Graft origin	EBV serology	Conditioning regimen	T-cell depletion	GvHD prophylaxis	EBV DNAemia whole blood copies/mL
5	9	F	AML	RELATED Sibling	BM	D?/R+	Busulfan-based myeloablative regimen	NO	Only CyA	Negative
9	4	M	ALL	MUD	BM	D-/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
10	11	M	AML	MUD	BM	D?/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
12	12	F	ALL	MUD	BM	D+/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MMF	Negative
13	5	M	AML	MUD	BM	D+/R+	Busulfan-based myeloablative regimen	*in vivo*	CyA + MTX	Negative
15	20	F	ALL	RELATED Sibling	BM	D+/R+	Busulfan-based myeloablative regimen	NO	Only CyA	Negative
16	1	F	ALL	RELATED Sibling	BM	D-/R+	Busulfan-based myeloablative regimen	NO	Only CyA	Negative

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; Pt, patient; M, male; F, female; MUD, matched unrelated donor; BM, bone marrow; D, donor; R, recipient; CyA, cyclosporine A; MTX, methotrexate; MMF, mycophenolate mofetil; GvHD, graft-versus-host disease.

Half of all patients (50%) underwent transplant for ALL. The donor type was a MUD for 10 allo-HSCT (55.6%) and a related donor type for 8 (44.4%) patients; in all transplants the donor was HLA-identical sibling. The source of stem cells was BM in 88.9% of transplants (16/18 patients). At the time of transplant, the EBV serostatus of D/R were: D+/R+ in 12/18 patients (66.6%) and D-/R+ in 3/18 patients (16.7%). The serology of the donor was not available in 3 cases (16.7%); all the respective recipients were EBV-seropositive. The conditioning regimen was of reduced intensity for 3 patients and MA for 15 patients. A total of 12 patients (66.7%) underwent *in vivo* T-cell depletion: all 10 MUD transplants and 2 out of 8 HLA-identical sibling transplants. On the basis of the patient-related and transplant-related characteristics, ATG rabbit (Thymoglobuline®, Genzyme, Cambridge, Massachusetts, USA) was administered in a total dose ranging from 6 to 9 mg/kg over 3 days, from day -4 to -2. In most cases, GvHD prophylaxis consisted of an association of cyclosporine A and methotrexate (n = 10).

The 18 paediatric allo-HSCT recipients were monitored for EBV infection for a median time of 12 months post-transplant (range, 2 - 13). All patients were EBV-seropositive at the time of transplantation. For 7 out of 18 patients (38.9%), WB samples tested negative for EBV DNA over the entire follow-up. Eleven patients (61.1%) developed a post-transplant EBV reactivation/reinfection: 6 patients who underwent allo-HSCT from MUD (60%, 6/10 patients) and 5 patients transplanted from related donor (62.5%, 5/8 patients). Among the 11 patients with an active post-transplant EBV infection, 3 patients (27.3%) showed low values of EBV DNAemia (< 10,000 copies/mL WB), whereas 8 patients (72.7%) showed values of EBV DNAemia equal or greater than 10,000 copies/mL WB. One out of 11 actively EBV infected patients (9.1%) developed an EBV-PTLD. The results of the virological follow-up are reported in Figure 12.

Figure 12: Results of the virological follow-up - Paediatric patients.



The median peak of viral load in patients with low and high values of EBV DNAemia was 2,727 copies/mL (range, 1,033 - 3,412 copies/mL) and 30,430 copies/mL (range, 10,763 - 2,011,688 copies/mL), respectively. The median time at which EBV DNAemia was first detected was 48 days (range, 25 - 143) post-transplant. The results obtained by virological monitoring, along with the range of EBV DNAemia values detected per patient, are reported in Table 5.

RISK FACTORS FOR DEVELOPING OF EBV INFECTION

Comparisons between patient-related and transplant-related characteristics and development of EBV infection were evaluated using chi-square test of independence. Patients who developed active post-transplant EBV infection were compared with not infected patients. In the analysis, adult and paediatric patients were considered as a unique population. P-values < 0.05 were considered statistically significant.

ADULT AND PAEDIATRIC STUDY POPULATION

RIC regimen in combination with *in vivo* T-cell depletion has been shown to be a risk factor for the developing of EBV infection rather than the primary disease; donor type; graft origin; *in vivo* T-cell depletion with ATG; acute GvHD and chronic GvHD. The analysis was reported in Table 7.

Table 7: Patient characteristics and risk factors for EBV infection - Adult and paediatric patients. Bold P-values are statistically significant

	Total	**EBV DNAemia NEGATIVE**	**EBV DNAemia POSITIVE**	**P value**
Number of patients (%)	51 (100)	25 (49)	26 (51)	
Primary disease				
ALL	16 (31.4)	7 (43.7)	9 (56.3)	0.873
AML	21 (41.1)	11 (52.4)	10 (47.6)	
CML	3 (5.9)	1 (33.3)	2 (66.7)	
Other *	11 (21.6)	6 (55.5)	5 (45.5)	
Donor type				
Unrelated (MUD)	36 (70.6)	19 (52.8)	17 (47.2)	0.406
Related (Sibling)	15 (29.4)	6 (40)	9 (60)	
Graft origin				
Bone marrow	24 (88.9)	13 (54.2)	11 (45.8)	0.563
Cord blood	3 (9.1)	2 (66.7)	1 (33.3)	
Peripheral blood	24 (11.1)	10 (41.7)	14 (58.3)	
Conditioning regimen				
Myeloablative#	45 (88.2)	25 (55.6)	20 (44.4)	**0.011**
Reduced-intensity	6 (11.8)	0	6 (100)	
In vivo T-cell depletion with ATG				
Yes	45 (88.2)	22 (48.9)	23 (51.1)	0.959
No	6 (11.8)	3 (50)	3 (50)	
Association - Conditioning regimen and ATG				
Myeloablative + ATG	39 (76.4)	22 (56.4)	17 (43.6)	**0.036**
Reduced-intensity + ATG	6 (11.8)	0	6 (100)	
Myeloablative Only	6 (11.8)	3 (50)	3 (50)	
CMV infection				
Yes	29 (56.9)	13 (44.8)	16 (55.2)	0.492
No	22 (43.1)	12 (54.5)	10 (45.5)	
Acute GvHD (< 100 days post-transplant) grading				
Absent	20 (39.2)	10 (50)	10 (50)	0.986
I	10 (19.6)	5 (50)	5 (50)	
$\geq$ II	21 (41.2)	10 (47.6)	11 (52.4)	
Chronic GvHD (> 100 days post-transplant) grading Data available for 50 patients				
Absent	41 (82)	21 (51.2)	20 (48.8)	0.331
Mild to severe	9 (18)	3 (33.3)	6 (66.7)	

ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; CML: chronic myeloid leukemia; MUD: matched-unrelated donor; ATG: antithymocyte immunoglobulin; CMV: cytomegalovirus; GvHD: graft-versus-host disease.

* severe aplastic anemia (n=1); beta-thalassemia major (n=2); Hodgkin lymphoma (n=1); anaplastic large cell lymphoma (n=1); multiple myeloma (n=2); diffuse large B-cell lymphoma (n=1); myeloid sarcoma (n=1); myelodysplastic syndrome (n=2).

#Myeloablative conditioning regimen includes both busulfan-based myeloablative regimen and total body irradiation-based myeloablative regimen.

2

SUITABILITY OF WHOLE BLOOD AS CLINICAL SPECIMEN TO MONITOR THE RISK OF DEVELOPING EBV-PTLD

Real-time PCR for EBV DNA quantification (EBV ELITe MGB^{TM} kit, ELITech Group, Turin, Italy) was performed on WB and PBMC samples, following the manufacturer's instructions.

Technical information

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-anticoagulated tubes. DNA was extracted from 200 µL of WB using the QIASymphony^{sp} instrument (Qiagen GmbH, Hilden, Germany), eluted in 90 µL of Elution Buffer and 20 µl were processed for EBV DNA quantification. DNA was extracted from $2x10^5$ PBMCs using the NucliSens easyMAG System (bioMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions; half of the eluated volume corresponding to $1x10^5$ PBMCs was used as input DNA in each real-time PCR. Amplification, detection and analysis were performed using the ABI PRISM 7300 platform (Life Technologies, Foster City, California, United States). The PCR assay targets a region of the EBNA-1p gene of EBV. The analytical sensitivity of the assay is 10 copies of target DNA per amplification reaction. The LLQ of the assay is 225 copies/mL WB and 11 copies/$1x10^5$ PBMCs. EBV DNA load was reported as quantities of copies/mL and copies/$1x10^5$ PBMCs.

The correlation between Log_{10} copies of EBV DNA in WB and PBMC samples was obtained using weighted Pearson correlation to take into account the different number of subjects' repeated measures.

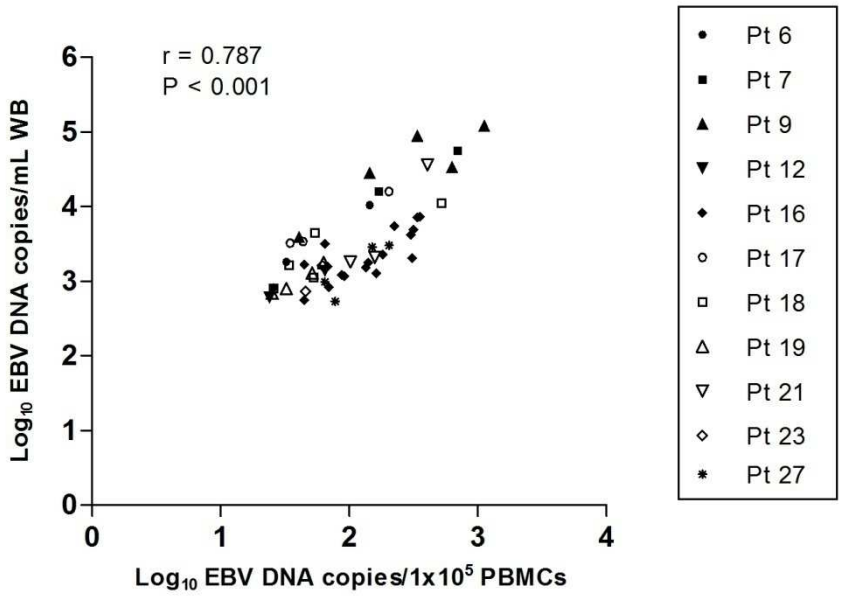
ADULT STUDY POPULATION

A total of 809 WB samples and 252 paired WB and PBMC samples were processed; the median number of paired WB and PBMC samples analyzed per patient was 18 (range, 3 - 33). Forty-nine out of 252 samples (19.5%) were negative and 178 samples (70.6%) were positive for EBV DNA both in WB and PBMC samples; 25 (9.9%) samples were discordant. Among the 178 positive samples: 50 samples (28.1%) were positive with a

quantitative result; 104 samples (58.4%) tested positive but below the LLQ of the PCR assay and 24 samples (13.5%) were positive with a quantitative result in WB and positive, but below the LLQ of the PCR assay in PBMC samples. The 50 samples that resulted positive with a quantitative result in both the two blood compartments were taken into consideration for the correlation analysis. The correlation coefficient obtained was r = 0.787 (P < 0.001) (Figure 13).

Figure 13: Correlation between EBV DNA levels in 50 WB and PBMC samples collected from adult allo-HSCT recipients.

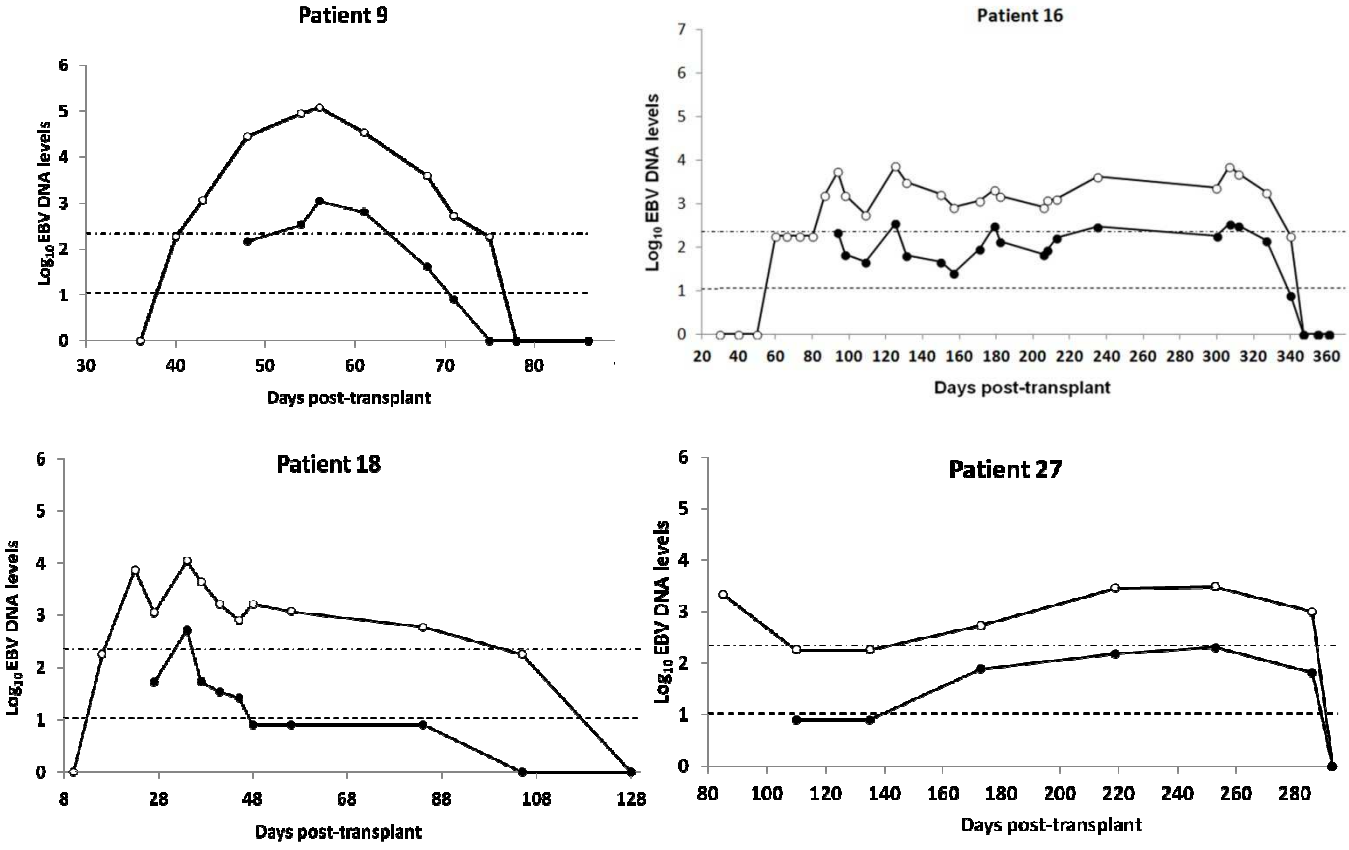
Pt: patient



EBV DNA kinetics in WB and PBMC samples were analyzed in all the 15 actively EBV infected patients. Representative EBV DNA kinetics in the two blood compartments of four out of 15 patients are reported in Figure 14.

Figure 14: Representative EBV DNA kinetics in WB and PBMC samples of four adult allo-HSCT recipients with active EBV infection.

The line (· – ·) indicates the LLQ of the real-time PCR assay for WB samples. The line (**– – –**) indicates the LLQ of the real-time PCR assay for PBMC samples. Log_{10} EBV DNA levels in WB and in PBMC samples were represented with open and filled circles, respectively.



PAEDIATRIC STUDY POPULATION

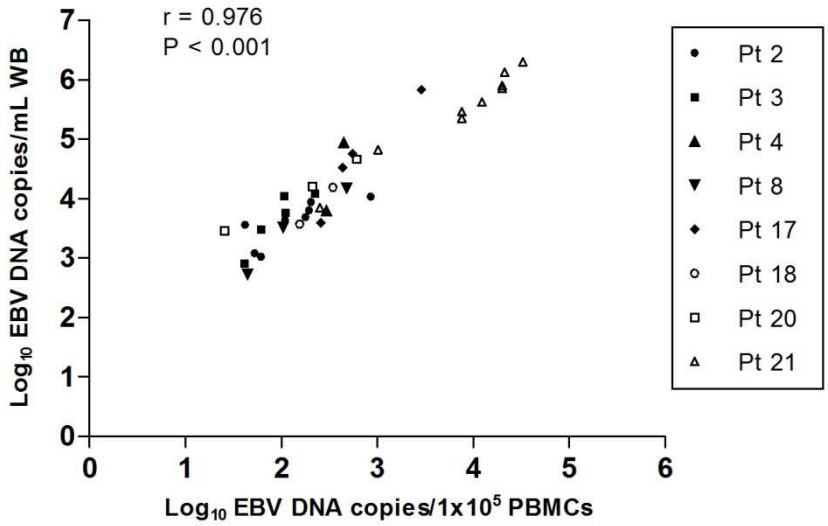
A total of 250 WB and 168 paired WB and PBMC samples were processed; the median number of paired WB and PBMC samples analyzed per patient was 13 (range, 3-31).

Fifty out of 168 samples (29.8%) were negative and 105 samples (62.5%) were positive for EBV DNA both in whole blood and PBMC samples; 13 (7.7%) samples were discordant. Among the 105 positive samples: 36 samples (34.3%) were positive with a

quantitative result; 43 samples (41%) tested positive but below the LLQ of the PCR assay and 26 samples (24.7%) were positive with a quantitative result in WB and positive but below the LLQ of the PCR assay in PBMC samples. The 36 samples that resulted positive with a quantitative result in both the two blood compartments were taken into consideration for the correlation analysis. The correlation coefficient obtained was r = 0.976 (P < 0.001) (Figure 15).

Figure 15: Correlation between EBV DNA levels in 36 WB and PBMC samples collected from paediatric allo-HSCT recipients.

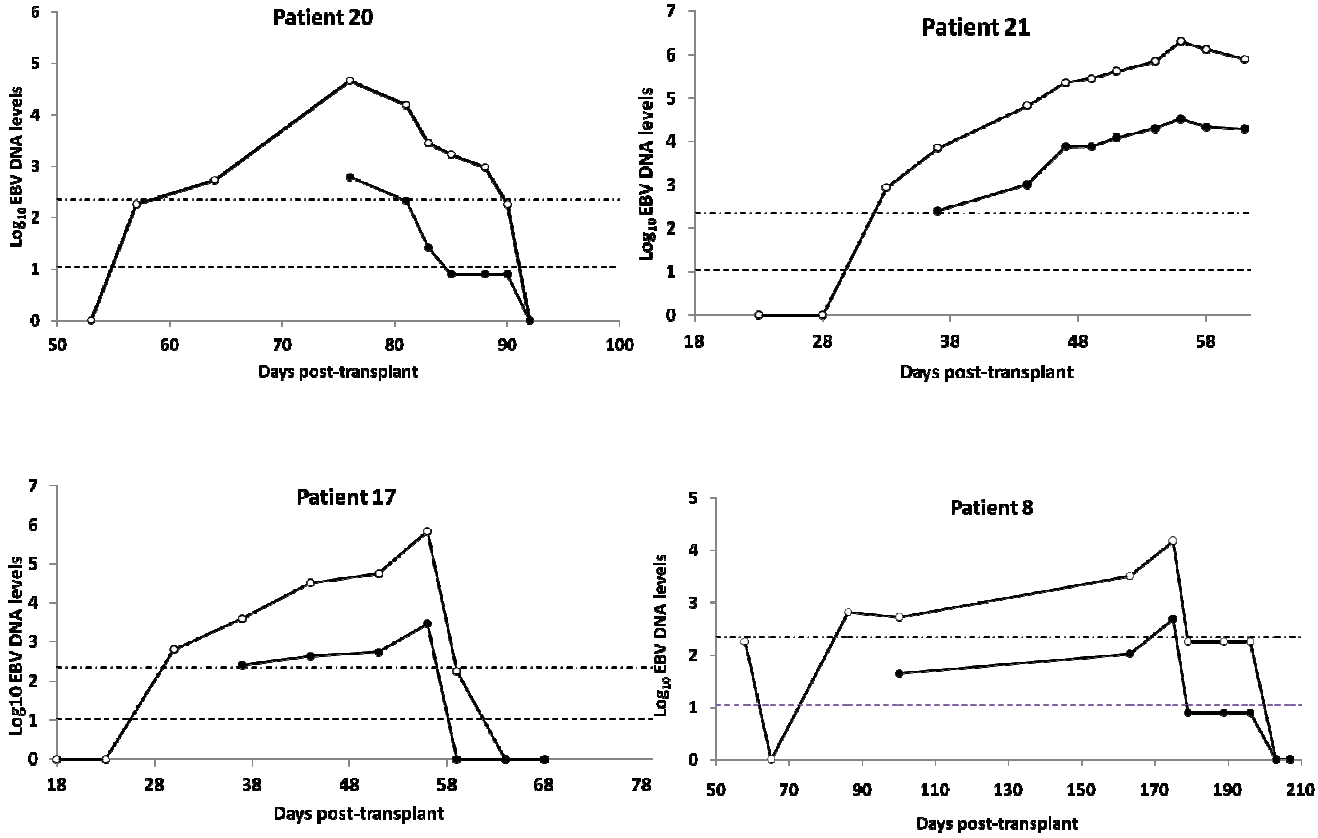
Pt: patient



EBV DNA kinetics in WB and PBMC samples were analyzed in all eleven patients. Representative EBV DNA kinetics in the two blood compartments of four out of 11 patients are reported in Figure 16.

Figure 16: Representative EBV DNA kinetics in WB and PBMC samples of four paediatric allo-HSCT recipients with active EBV infection.

The line (· ━ ·) indicates the LLQ of the real-time PCR assay for WB samples. The line (┅) indicates the LLQ of the real-time PCR assay for PBMC samples. Log_{10} EBV DNA levels in WB and in PBMC samples were represented with open and filled circles, respectively.



3

COMBINED VIROLOGICAL-IMMUNOLOGICAL MONITORING OF EBV INFECTION AS A POSSIBLE PREDICTIVE MARKER OF EBV-RELATED COMPLICATIONS

The immunological monitoring was carried out using EliSpot assay (EliSpot Interferon-γ Basis Kit; GenID GmbH, Strasburg, Germany).

Technical information

Blood samples were collected in sodium citrate-treated tubes. PBMCs were isolated by density gradient centrifugation using Ficoll Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). An aliquot of $2x10^5$ PBMCs was stimulated separately with phytohemagglutinin (PHA-P) at 5 µg/ml, with latent and lytic viral antigens at 5 µg/ml or culture medium only (negative control), for 20-24 hours in a CO_2 incubator. Two commercially available latent and lytic EBV-specific peptide mix were used (GenID GmbH, Strasburg, Germany). In particular, peptide pools derived from Epstein-Barr nuclear antigens ($EBNA_{pp}$) such as $EBNA3A_{pp}$ (amino acid (AA) 158-166, 325-333, 379-387, 458-466, 603-611) and $EBNA3C_{pp}$ (AA 258-266, 281-290), latent membrane protein 2-derived single peptide ($LMP2_p$, AA 426-434), $BRLF1_{pp}$ (AA 148-156, 134-143, 28-37), $BMLF1_{pp}$ (AA 259-267, 280-288) and $BZLF1_p$ (AA 190-197) were used. In response to the antigen, T cells specifically release INF-γ, which is then bound by the antibody coated to the well. Cytokine release is visualized by an enzyme-labelled detection antibody. The end results are blue spots, each of which represents the area of one cell secreting several molecules of INF-γ. Coloured spots were counted using a computer-assisted image analysis system (AID EliSpot Reader System, Strassberg, Germany). The EliSpot results were interpreted as reported in Table 8.

Table 8: Interpretation of EliSpot results.

EBV LYTIC or/and LATENT ANTIGENS *minus* NEGATIVE CONTROL (SFCs/2x10^5 PBMCs)	**MITOGEN (PHA-P) *minus* NEGATIVE CONTROL (SFCs/2x10^5 PBMCs)**	**EliSpot RESULT**	**INTERPRETATION OF RESULTS**
$\leq$ 5	$\leq$ 50	Indeterminate	CMI NOT DETECTED
$\leq$ 5	$\geq$ 50	Negative	EBV-specific CMI NOT DETECTED
$\geq$ 5	$\geq$ 50	Positive	EBV- specific CMI DETECTED

PHA: phytohemagglutinin; SFCs: spot forming cells; CMI: cell-mediated immunity.

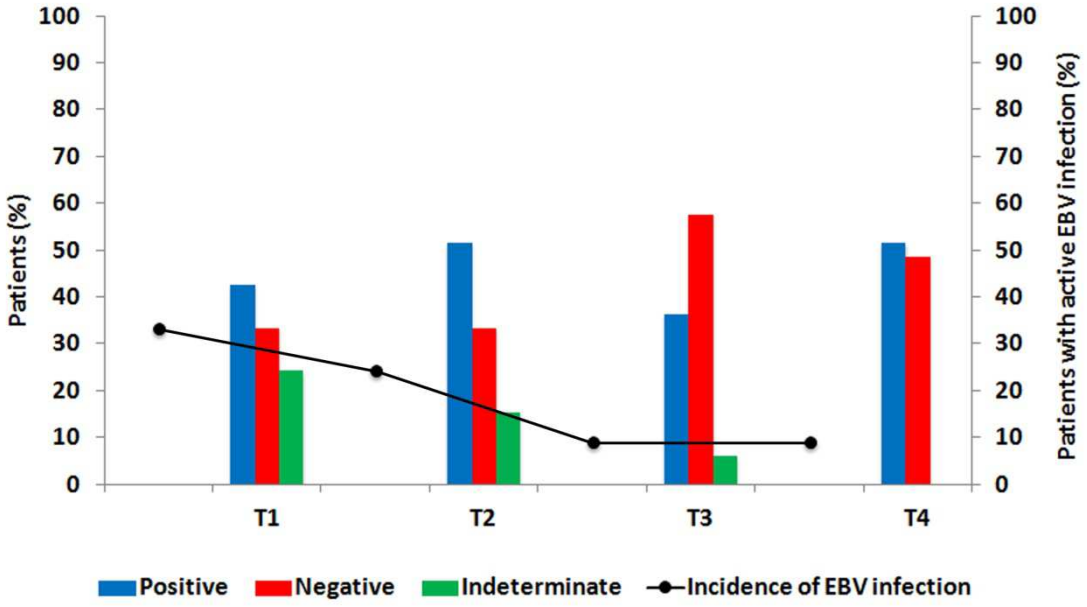
The mitogen stimulation and the negative control were included to determine general T-cell responsiveness (PHA-P $\geq$ 50 spot forming cells (SFCs)/2x10^5 PBMCs) and background ($\leq$ 10 SFCs/2x10^5 PBMCs), respectively. Cellular immunity to EBV in healthy EBV-seropositive individuals was also evaluated to check each assay run.

According to the study design, EliSpot assay was performed at four fixed points after transplant (+60, +100, +180, +360 day) for all patients and when the patients exceeded 10,000 copies/mL WB at the next visit.

ADULT STUDY POPULATION

EliSpot results were correlated with the incidence of EBV infection (Figure 17).

Figure 17: EliSpot results and incidence of EBV infection - Adult patients.
T1 (+60 day), T2 (+100 day), T3 (+180 day) and T4 (+360 day).

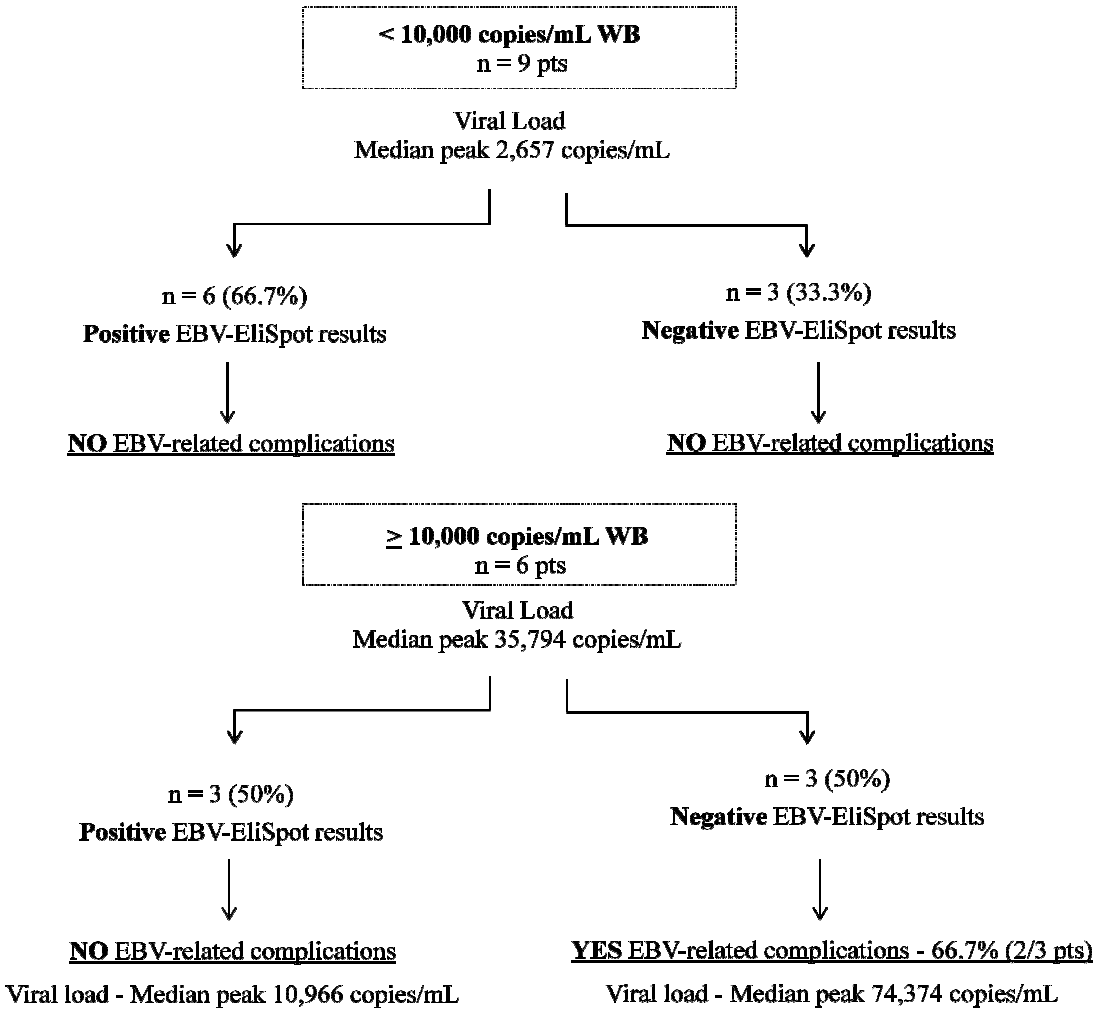


Moreover, the results of immunological monitoring obtained in patients with active EBV infection were analyzed; the patients were grouped on the basis of the viral load detected (< 10,000 copies/mL WB and ≥ 10,000 copies/mL WB) (Figure 18).

Figure 18: Results of the immunological monitoring of EBV infection and clinical outcome - Adult patients.

The EliSpot results obtained during active EBV infection or at the time-point after the onset of infection were taken into account.

pts: patients

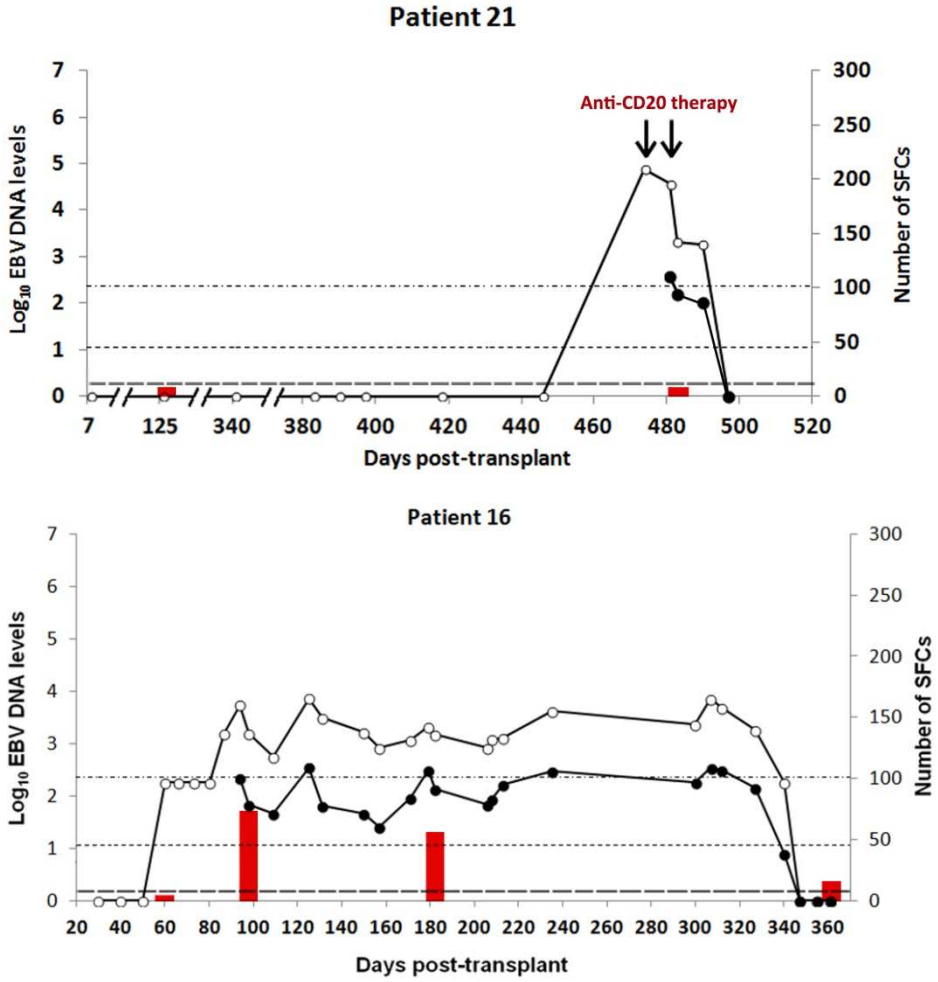


Representative combined virological-immunological monitoring of EBV infection of 2 out of 15 actively EBV infected patients are reported in Figure 19.

Figure 19: Results of combined virological-immunological monitoring of EBV infection of two adult patients.

The line (· – ·) indicates the LLQ of the real-time PCR assay for WB samples. The line (…..) indicates the LLQ of the real-time PCR assay for PBMC samples. Log_{10} EBV DNA levels in WB and in PBMC samples were represented with open and filled circles, respectively. The line (– – –) indicates the EliSpot assay cut-off for positive response to EBV ($\geq$ 5 SFCs/2x10^5 PBMCs, calculated using the formula "sample – negative control").

The bar (▮) indicates the EliSpot result.



PAEDIATRIC STUDY POPULATION

The results of combined virological-immunological monitoring of EBV infection in paediatric patients were analyzed in a fashion similar to the adult study population (Figures 20-22).

Figure 20: EliSpot results and incidence of EBV infection - Paediatric patients.
T1 (+60 day), T2 (+100 day), T3 (+180 day) and T4 (+360 day).

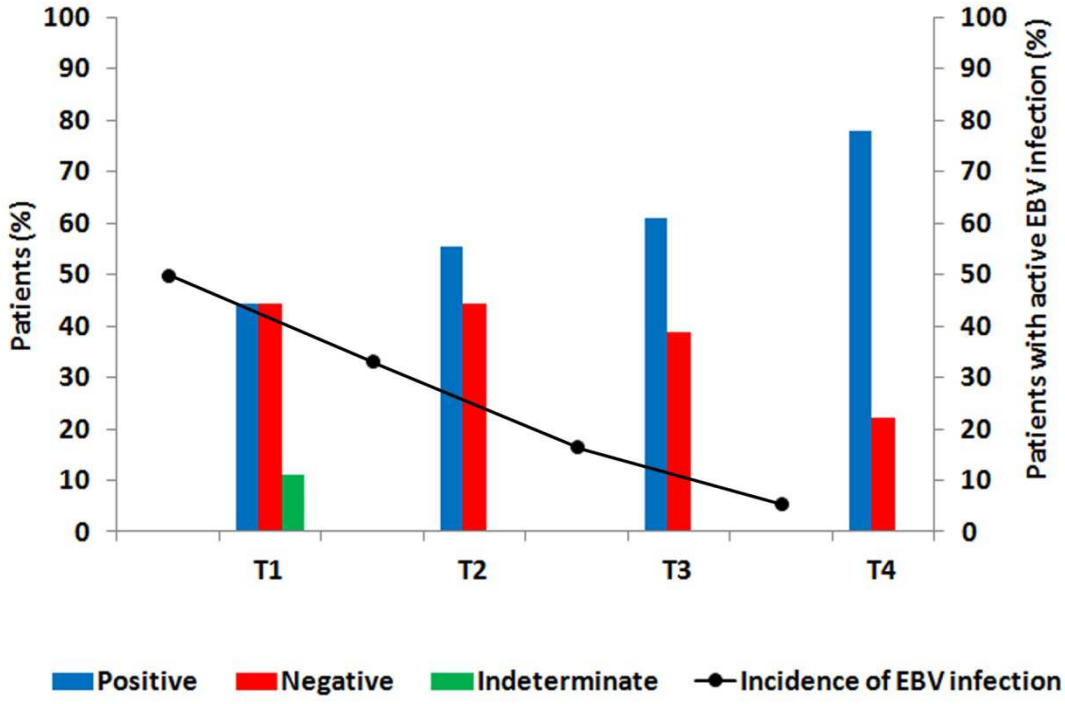


Figure 21: Results of the immunological monitoring of EBV-infection and clinical outcome - Paediatric patients.

The EliSpot results obtained during active EBV infection or at the time-point after the onset of infection were taken into account.

pts: patients

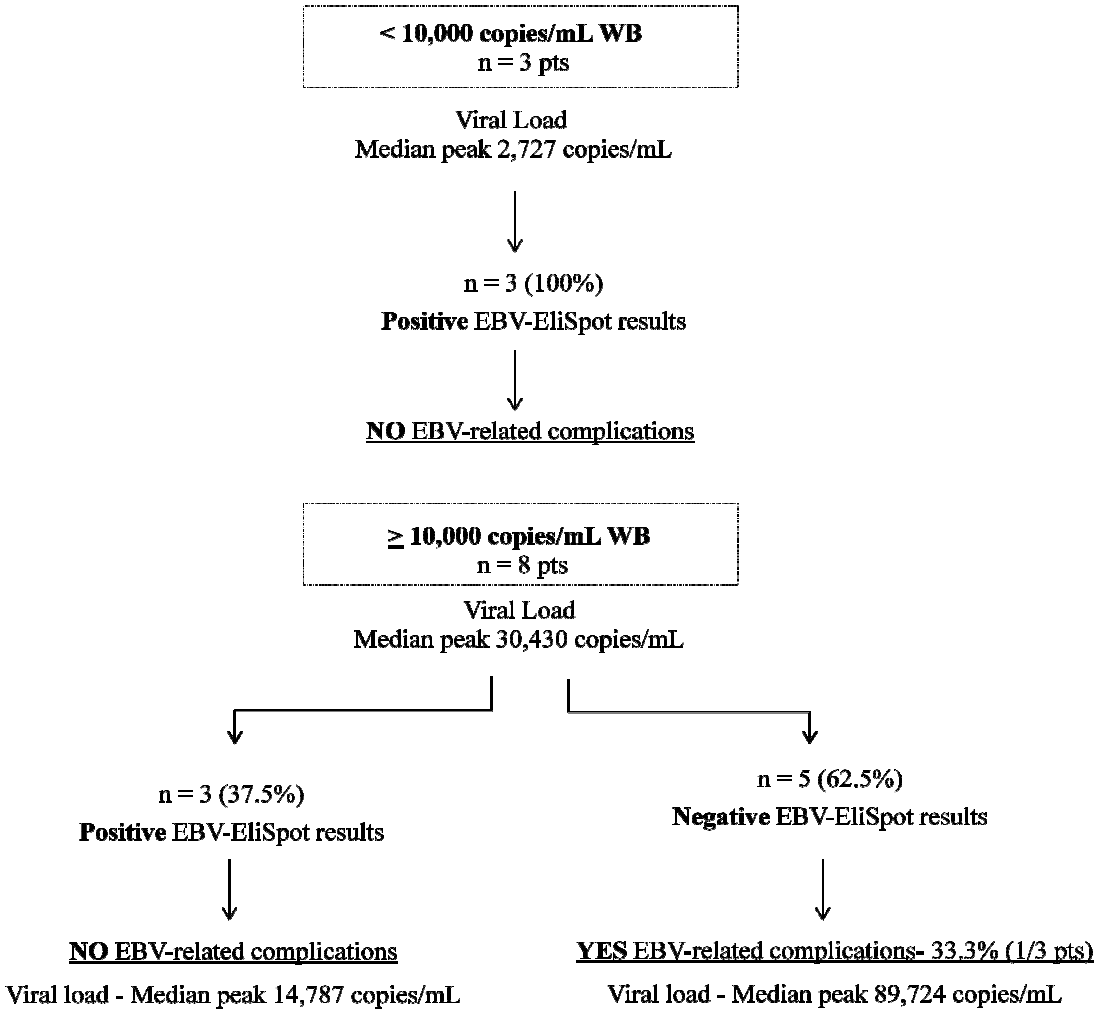
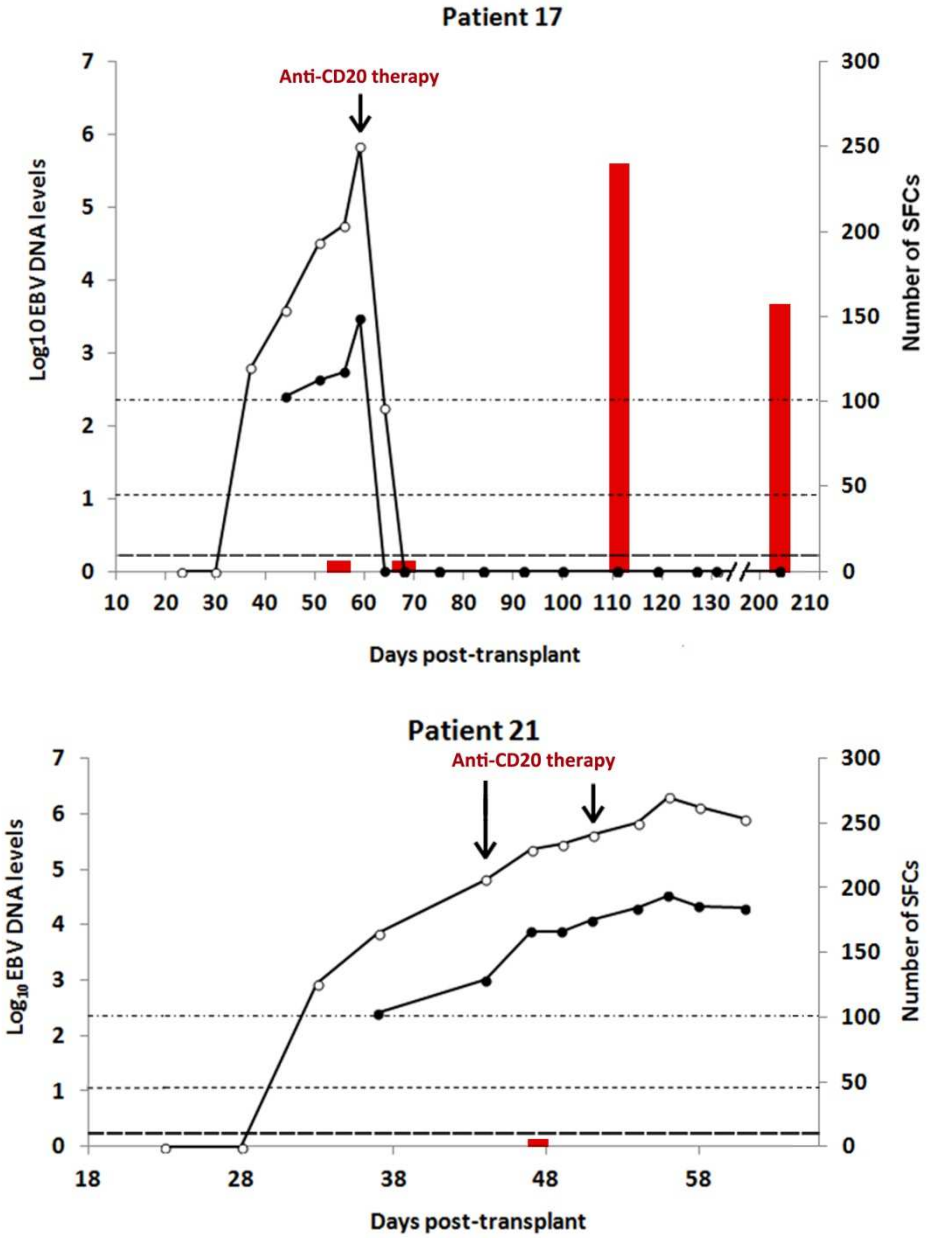


Figure 22: Results of combined virological-immunological monitoring of EBV infection of two paediatric patients.

The line (· – ·) indicates the LLQ of the real-time PCR assay for WB samples. The line (…..) indicates the LLQ of the real-time PCR assay for PBMC samples. Log_{10} EBV DNA levels in WB and in PBMC samples were represented with open and filled circles, respectively. The line (– – –) indicates the EliSpot assay cut-off for positive response to EBV ($\geq$ 5 SFCs/2x10^5 PBLs, calculated using the formula "sample – negative control").

The bar (▮) indicates the EliSpot result.



ASSOCIATION OF EBV-SPECIFIC T-CELL RESPONSES WITH PEAK VIRAL LOAD

The association between EBV-specific CMI and EBV DNAemia peak were evaluated using Mann-Whitney test. Among patients who developed an active EBV infection, patients who had a positive EBV-EliSpot result - i.e. patients with detectable EBV-specific CMI - were compared with patients who resulted EBV-EliSpot negative - i.e. patients without EBV-specific CMI. In the analysis, adult and paediatric patients were considered as a unique population. P-values $\leq$ 0.05 were considered statistically significant.

ADULT AND PAEDIATRIC STUDY POPULATION

Median peak levels of EBV DNAemia were significantly higher for patients with negative EBV-EliSpot result (6 adult and 5 paediatric patients) than for patients with positive EBV-EliSpot result (9 adult and 6 paediatric patients) during active or at time-point after the onset of EBV infection (P = 0.013) (Table 9).

Table 9: Association of EBV-specific CMI with peak viral load.

EBV-EliSpot **POSITIVE**	**EBV-EliSpot** **NEGATIVE**
Peak viral load - copies/mL WB	**Peak viral load - copies/mL WB**
3,035	1,703
3,174	3,386
1,038	1,769
2,657	55,769
7,279	119,039
1,611	74,374
3,412	12,395
1,033	89,724
2,727	683,294
10,521	45,548
15,820	2,011,688
10,966	
10,763	
14,787	
15,312	
n = 15; **Median = 3,412**	n = 11; **Median = 55,769**
Mann-Whitney test: z = 2.465 **P = 0.013**	

4

THE MANAGEMENT OF EBV INFECTION AND PTLD PREVENTION

The management of EBV infection for patients with any value of EBV DNAemia ≥ 10,000 copies/mL was defined at individual patient level. Proven EBV-PTLD was defined according to the European Conference on Infections in Leukemia (ECIL) guidelines (37). Furthermore, cases of proven EBV-PTLD were classified according to the WHO criteria (34).

ADULT STUDY POPULATION

Among the six patients with high viral load, the three patients with detectable EBV-specific CMI (patients number 17, 18 and 6) controlled EBV replication spontaneously and were all completely asymptomatic, while the three patients without EBV-specific CMI (patients number 7, 9 and 21) received anti-CD20 monoclonal antibody rituximab (2-4 administrations, at dose of 375 mg/m^2/week) and 2 of them (2/3; 66.7%) developed EBV-related complications such as mononucleosis-like syndrome (fever and pharyngitis) at +474 day (patient n. 21) and EBV-PTLD with nodal involvement at +65 day (patient n. 9) post-transplant. For all patients, the first dose of anti-CD20 therapy was administered at the peak of EBV DNA levels in WB. For two out of 3 patients, the peak of EBV DNA levels were reached simultaneously in both the two blood compartments; the median EBV DNA levels was equal to 74,374 copies/mL WB and to 902 copies/1x10^5 PBMCs. For the remaining patient (patient number 21), the peak of viral load (74,374 copies/mL) was coincident with the first positivity; then the respective EBV DNA levels in PBMCs was not available. During the treatment, a decrease of EBV DNA levels in both blood compartments was observed in all patients who achieved negativity at the end of the anti-CD20 therapy. Nevertheless, in one patient (33.3%; patient number 9), despite pre-emptive anti-CD20 monoclonal antibody administration, the progression of EBV infection to overt disease was observed. The successful management of EBV-PTLD in this patient by virological-immunological monitoring of EBV infection, prompt diagnosis and early treatment is described in detail in a published Brief Communication (Transpl Immunol. 2016;34:60-64) that is

provided in Part VII - Annexes. In all the nine patients who maintained viral loads < 10,000 copies/mL WB, EBV clearance occurred spontaneously and the EBV infection was asymptomatic. It is recalled that the majority of the patients (6/9; 66.7%) presented EBV-specific cellular immune responses during active EBV infection or at the first time-point after EBV DNAemia and 3 out of 9 patients (33.3%) presented general T-cell responsiveness.

Virological, immunological and clinical data of adult patients with active EBV infection are summarized in Table 10.

Table 10: Fifteen adult patients with active EBV infection - Virological, immunological and clinical data.

Pt	EBV serostatus D/R	Onset of EBV infection Days post-TX	EBV DNAemia Peak copies/mL WB	EBV EliSpot result	Management of EBV infection	EBV-related complications	Outcome
9	D?/R+	40	119,039	Negative	Anti-CD20 therapy	Nodal polymorphic and monomorphic DLBCL-like	Alive
21	D?/R+	474	74,374	Negative	Anti-CD20 therapy	Mononucleosis-like syndrome (fever and pharyngitis)	Alive
7	D+/R+	62	55,769	Negative	Anti-CD20 therapy	NO	Alive
17	D+/R+	52	15,820	Positive	Spontaneous Clearance	NO	Alive
18	D+/R+	20	10,966	Positive	Spontaneous Clearance	NO	Dead Relapse of the original disease
6	D+/R+	42	10,521	Positive	Spontaneous Clearance	NO	Alive
16	D?/R+	87	7,279	Positive	Spontaneous Clearance	NO	Dead Relapse of the original disease
5	D+/R+	53	3,386	Negative	Spontaneous Clearance	NO	Alive
2	D+/R+	35	3,147	Positive	Spontaneous Clearance	NO	Alive
27	D-/R+	85	3,035	Positive	Spontaneous Clearance	NO	Alive
12	D+/R+	47	2,657	Positive	Spontaneous Clearance	NO	Alive
19	D+/R+	35	1,769	Negative	Spontaneous Clearance	NO	Alive
34	D-/R+	95	1,703	Negative	Spontaneous Clearance	NO	Alive
23	D+/R+	55	1,611	Positive	Spontaneous Clearance	NO	Alive
4	D+/R-	40	1,038	Positive	Spontaneous Clearance	NO	Dead Relapse of the original disease

Pt, patient; D, donor; R, recipient; TX, transplant; DLBCL: diffuse large B-cell lymphoma.

PAEDIATRIC STUDY POPULATION

The 3 patients with EBV DNA load $\geq$ 10,000 copies/mL WB and with detectable EBV-specific CMI controlled EBV replication spontaneously and were all completely asymptomatic. Among the five patients without EBV-specific CMI, 2 (40%; patient number 3 and 20) cleared spontaneously EBV infection and had an asymptomatic infection; while 3 (60%; patient number 4, 17 and 21) received anti-CD20 monoclonal antibody rituximab (1-2 administrations, at dose of 375 mg/m^2/week). Among the latter 3 patients, for 2 patients (66.7%; patient number 4 and 17) the first dose of anti-CD20 therapy was administered at the peak of EBV DNA levels that were reached simultaneously in both the two blood compartments; the median EBV DNA levels was equal to 386,509 copies/mL WB and to 1,678 copies/1x10^5 PBMCs. A decrease of EBV DNA levels in both blood compartments was observed during the treatment and at the end of the therapy they achieved EBV DNA negativity. Regarding the remaining patient (33.3%; patient number 21), he developed an EBV-PTLD with both nodal and extra-nodal involvement, i.e. gastrointestinal tract (stomach and large bowel), at 45 days post-transplant. In particular, a sudden increase in EBV DNA levels in both WB and PBMCs samples was observed during the weekly virological monitoring and anti-CD20 monoclonal antibody rituximab was immediately administrated due to high viral load values (65,881 copies/mL WB and 1,021 copies/1x10^5 PBMCs), but the patient had clinically overt PTLD. During the treatment - after 2 rituximab administrations - PCR assay revealed an increase in EBV DNA up to a peak of viral load of 2,011,688 copies/mL WB and 33,263 copies/1x10^5 PBMCs (+59 day post-transplant) and the patient showed persistence of symptoms and signs of PTLD. Furthermore, the patient's clinical condition rapidly worsened, until reaching renal and respiratory failures. The patient died of multi-organ failure at +61 days post-transplant.

In all the three patients who maintained viral loads < 10,000 copies/mL WB, a positive response to EBV was detected by EliSpot assay, EBV clearance occurred spontaneously and the EBV infection was asymptomatic.

Virological, immunological and clinical data of paediatric patients with active EBV infection are summarized in Table 11.

Table 11: Eleven paediatric patients with active EBV infection - Virological, immunological and clinical data.

Pt	EBV serostatus D/R	Onset of EBV infection Days post-TX	EBV DNAemia Peak copies/mL WB	EBV EliSpot result	Management of EBV infection	EBV-related complications	Outcome
21	D+/R+	33	2,011,688	Positive	Anti-CD20 therapy	- Nodal monomorphic DLBCL-like - Extra-nodal PTLD involving stomach and large bowel	Dead Multi-organ failure
17	D+/R+	44	683,294	Negative	Anti-CD20 therapy	NO	Alive
4	D+/R+	28	89,724	Negative	Anti-CD20 therapy	NO	Alive
20	D?/R+	76	45,548	Negative	Spontaneous Clearance	NO	Alive
18	D+/R+	25	15.312	Positive	Spontaneous Clearance	NO	Alive
8	D-/R+	158	14,787	Positive	Spontaneous Clearance	NO	Alive
3	D+/R+	56	12,395	Negative	Spontaneous Clearance	NO	Dead Renal and respiratory failure
2	D?/R+	143	10,763	Positive	Spontaneous Clearance	NO	Alive
1	D+/R+	85	3,412	Positive	Spontaneous Clearance	NO	Alive
19	D+/R+	48	2,727	Positive	Spontaneous Clearance	NO	Alive
7	D+/R+	40	1,033	Positive	Spontaneous Clearance	NO	Dead Pneumonia

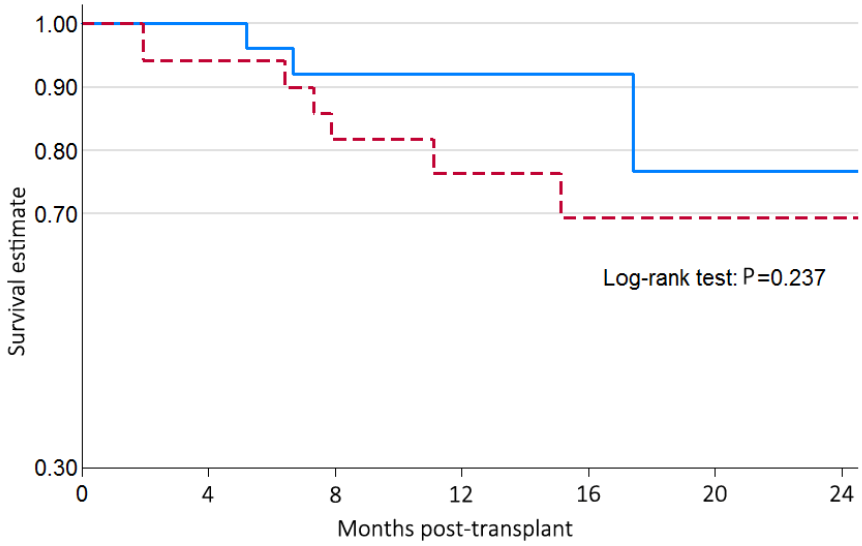
Pt, patient; D, donor; R, recipient; TX, transplant; DLBCL: diffuse large B-cell lymphoma.

CLINICAL OUTCOME - ADULT AND PAEDIATRIC STUDY POPULATION

Survival of patients who underwent transplant was defined as the time (months) from transplantation to either death or last observation. In particular, the median clinical follow-up was of 15 months after allo-HSCT (range, 2-24). Survival estimates were obtained using the Kaplan-Meier approach. Comparison between groups - actively EBV infected *versus* EBV not infected patients - was performed using the log-rank test (Figure 23).

Figure 23: Kaplan-Meier survival curves of transplanted patients by EBV reactivation.

The dotted line represents patients who developed active EBV infection. The solid line represents patients not EBV infected.



The 2-year survival estimate was 0.694 in actively EBV infected patients and 0.768 in not infected patients (log-rank test: chi2=1.40, p = 0.237)

In our cohort, a total of 9 patients died (17.3%; 9/52). Specifically, 6 out of 33 (18.2%) adult patients died at a median time of 7 months (range, 5-11) after allo-HSCT; the causes of death were relapse of the original disease (n = 4), sepsis (n = 1) and bacterial pneumonia (imaging diagnosis; n = 1). Three out of 18 paediatric patients (16.7%) died

at a median time of 6.4 months (range, 2-15) after allo-HSCT; the causes of death were multi-organ failure (n=1), renal and respiratory failure (n = 1) and bacterial pneumonia (imaging diagnosis; n = 1).

Part V

CONCLUSION AND PROSPECTIVES

EBV-PTLD is an uncommon, but frequently fatal, complication after allo-HSCT and prompt diagnosis is necessary due to the rapid and often disseminated nature of the disease (40,49,77). EBV-PTLD may present with a diverse spectrum of clinical symptoms and signs, underscoring the need for a high index of suspicion in making the diagnosis (79). In the past, mortality rates due to EBV-driven disease was greater than 90%. In fact, its treatment was limited to reduction of immunosuppression, chemotherapy or unselected donor lymphocytes infusion. Current treatment strategies, such as anti-CD20 monoclonal antibody rituximab and EBV-specific cytotoxic T lymphocytes therapy, have significantly reduced the risk of death in established EBV-PTLD after stem cell transplant (80). Therefore, because the clinical presentation of PTLD is rather non-specific and because early diagnosis and treatment result in better outcome, there is great interest in developing tests to predict the development of the disease (79). In the present PhD project, 51 allo-HSCT recipients - 33 adult and 18 paediatric - underwent combined virological-immunological monitoring for EBV infection during post-transplant period. Virological monitoring of EBV infection was performed for all patients by processing WB samples using a quantitative real-time PCR. EBV DNA quantification in both WB and in PBMC samples was adopted for patients who developed an active EBV infection. Immunological monitoring was carried out with INF-γ EliSpot that is an increasingly diffused clinical laboratory technique for monitoring the cellular immune responses to infections - with IFN-γ production by T cells having been shown to correlate with functional immunity (81).

As reported in previous studies (67,68), we observed that active EBV infection is a frequent event after transplant: the overall incidence of EBV infection in our cohort was equal to 51% (45.5% in adult and 61.1% in paediatric recipients). Moreover, EBV infection with high viral load was observed in 53.8% of the actively infected patients (in 40% of the adult and in 72.7% of paediatric recipients). As expected, the onset of EBV infection was observed during the early post-engraftment phase; in particular at a median time of 52 and 48 days post-transplant in adult and paediatric patients, respectively. Nevertheless, EBV DNAemia was first detected after 1 year post-transplant in 1 adult patient (unrelated CB transplant), who developed a symptomatic EBV infection at +474 day (patient number 21). The finding of late-onset EBV

infection underlines the need to continue virological monitoring for patients who may be at high risk, e.g. as long as GVHD prophylaxis or treatments are administered.

We identified that RIC HSCT in combination with selective depletion of recipient T cells with ATG relative to B-cells, was associated with higher frequencies of EBV infection, pointing to the same result recognized by other studies (82,83), including one of our previous studies entitled "Prospective Epstein-Barr virus-related post-transplant lymphoproliferative disorder prevention program in paediatric allogeneic hematopoietic stem cell transplant: virological monitoring and first-line treatment" (84) (Transpl Infect Dis. 2016;18:44-54; see paper in Part VII - Annexes). This probably reflects the profound immunosuppression following RIC HSCT, together with the incomplete ablation of recipient-derived B-cells. In our cohort of patients, we were not able to evaluate the contribution of EBV serology mismatch between donor and recipient to developing of post-transplant EBV infection because donor EBV serology was available for 42 out of 51 EBV seropositive patients (n = 7 D-/R+ and 35 D+/R+). The recipients' EBV serostatus at the time of transplant was also not taken into consideration for the analysis, because only one patient resulted EBV-seronegative (D+/R-; adult patient number 4). Finally, none of the patients underwent splenectomy, therefore comparison between this clinical parameter and EBV infection could not be evaluated.

Measurement of EBV DNA load by quantitative real-time PCR assays can be a sensitive aid to PTLD diagnosis, but unfortunately it is not always specific for disease onset (73). In general, assays using PBMCs are the most sensitive and predict EBV disease at the earliest time, but owing to technical aspects, it would be preferable to test EBV DNA load in WB samples (73, 85). In agreement with previous studies (84,86,87), in both the adult and paediatric study populations, we obtained a significant correlation between EBV DNA levels in WB and PBMC samples ($r = 0.787$ and $r = 0.976$, $P < 0.001$, respectively). Moreover, a similar kinetics of EBV DNA load in the two different blood compartments of the patients was observed: peak DNA levels were reached simultaneously and the times of DNA clearance were comparable. In addition, both specimen types appeared to be equally informative to assess the risk of patients to develop PLTD in the clinical setting. In fact, in the patients, EBV infection was managed with the use of anti-CD20 monoclonal antibody rituximab at the peak of DNA levels or when the EBV DNA levels exceed the EBV DNA threshold levels for

preemptive therapy defined in GITMO guidelines (i.e. 10,000 copies/mL WB and > 1,000 copies/1x10^5 PBMCs) that was occurred simultaneously in both the blood compartments. Therefore, among the study population on the basis of EBV DNA levels detected in WB samples, we were able to identify 5 patients (19.2%) eligible for pre-emptive therapy. In the 80% of cases (4/5 patients), the first-line treatment for EBV-related disease was effective in controlling viral proliferation and avoiding progression into EBV-PTLD. The remaining patient, despite the administration of pre-emptive anti-CD20 therapy developed an EBV-PTLD with nodal involvement. However, the early use of the anti-CD20 monoclonal antibody has proven to be a safe and effective treatment strategy for proven EBV-PTLD. In fact, a decrease in EBV-DNA load of 2 log of magnitude in the first two weeks of treatment was observed and EBV infection was cleared at the end of the therapy. A complete disease remission, two months after the EBV-PTLD diagnosis and four doses of anti-CD20 monoclonal antibody was observed and both the blood compartments tested negative for EBV DNA. EBV-PTLD persists in remission 10 months following the initial diagnosis. Another case of EBV-PTLD in the early post-transplant period was observed, but with an aggressive clinical course. In particular, this paediatric patient showed early viral reactivation followed by rapid progression to full-blown PTLD: before preemptive therapy could be started, the aforementioned patient already had clinical signs of overt PTLD. The patient developed an EBV-PTLD with both nodal and extra-nodal involvement at 45 days post-transplant, no response ensued to rituximab administrations and the patient died 15 days after EBV-PTLD diagnosis. These findings are in agreement with the literature data. In fact, in a recent comprehensive review of reported cases regarding the outcome of treatment of EBV-PTLD in HSCT recipients, the successful prevention of PTLD was reported in 89.7% of the patients receiving rituximab as preemptive therapy (50). Furthermore, in several studies, rituximab administered for treatment purposes yielded a 40-68% response rate (47-49). Finally, the occurrence of rapid progression to lethal PTLD was also reported by other investigators (78). Accordingly, the overall frequency of biopsy-proven EBV-PTLD in our cohort was 3.9% (2/51 patients), which is in line with other recent reports (41, 88).

The pattern of general and EBV-specific T-cell reconstitution in our cohort was analyzed. We observed that the higher number of indeterminate results was present

during the early post-transplant period, that over time the immune response became more sustained and that concurrently the percentage of patients with active EBV infection clearly decreased. Furthermore, as observed by other authors (89), compared to adult recipients, paediatric had faster CMI reconstitution. In fact, all paediatric patients developed general CMI within 100 days post-transplant *versus* 360 days post-transplant of the adult recipients. In our cohort, the 42.4% of the adult and the 44.4% of the paediatric patients showed EBV-specific CMI at 60 days post-transplant and it may be due to the rate of EBV infection occurred during the first two months post-transplant. In fact, it has been suggested that the occurrence of EBV DNA positivity may implement the responsiveness of the patient's immune system (81); in our cohort, the 33% and 50% of adult and paediatric patients developed respectively an active EBV infection within 60 days post-transplant. Moreover, as reported by other studies (81,90), we observed that EBV-specific T-cell responses were not constant; 29.4% of the adult patients with a positive EBV-EliSpot result subsequently had a negative result.

EliSpot responses to latent and lytic EBV-specific peptide mix were evaluated for each patient. In healthy individuals the hierarchy of T-cell responses against the different latent and lytic EBV antigens is well established (91), whereas the analysis of the EBV-specific T-cell responses in patients after transplant remains largely unexplored (74). In our cohort, we did not observe a distinct pattern of distribution of EBV-specific T-cell responses to lytic and latent antigens (data not shown). A similar result was obtained in a solid organ transplant setting, where the authors reported that the distribution of EBV-specific T-cell responses observed in transplant recipients was altered compared to that observed in healthy individuals (92). The correlation between EBV-specific T-cell responses and EBV infection, specifically in relation to viral load and clinical course was also studied. The lack of EBV-specific T-cell immune responses during the active EBV infection or at the time-point after the onset of infection was associated with a significantly higher median peak level of EBV DNA in blood and a greater severity of EBV infection. In fact, the 54.5% of the patients without detectable EBV-specific CMI needed anti-CD20 therapy because they had not been able to control EBV replication spontaneously and the 27.2% developed EBV-related complications including, as previously reported, a lethal PTLD. On the contrary, all patients who had a detectable EBV-specific CMI controlled EBV replication spontaneously and were all completely

asymptomatic. As reported by other studies (89,93), these findings suggest that post-transplant EBV-specific CMI is a critical determinant controlling the EBV infection and consequently the virus-related complications.
In our cohort, the majority cause of death was the relapse of the original disease, accounting for the 44.4% of cases. As other authors, we did not observe detrimental effect of EBV active infection on survival patient (69,94).

In conclusion, the present study suggests that a close EBV DNAemia monitoring may be a useful strategy to control EBV-related PTLD in high risk patients. In fact, WB proved to be a suitable clinical specimen to monitor EBV DNA load after allo-HSCT for the management of post-transplant EBV infection and PTLD prevention. Moreover, this study confirms that RIC in combination with *in vivo* T-cell depletion is a particularly important risk factor for the developing of EBV infection. Finally, our data showed the potential usefulness of measuring EBV blood replication combined with the specific viral cellular-immunity in allo-HSCT recipients in order to identify the patients at higher risk for EBV-PTLD who would benefit from appropriate pre-emptive interventions.

In the light of the results obtained, we propose in our Centre at St. Orsola-Malpighi University Hospital, the following combined virological-immunological monitoring schedule in order to improve the management of EBV infection in those patients at higher risk of developing EBV-related complications. When EBV-DNA load is over 10,000 copies/mL WB and in the absence of EBV-related signs or symptoms, the EBV-EliSpot assay could be required to evaluate EBV-specific T-cell recovery kinetics. EBV-EliSpot result should be an important second parameter for identifying patients who may cleared spontaneously EBV DNA load (positive EBV-EliSpot result) or who will benefit most from pre-emptive interventions (negative EBV-EliSpot result). In the latter case, a closer virological monitoring should be performed and the immunological assay should be repeated a month later in order to detect the reconstitution or not of EBV-specific CMI. Such a strategy requires confirmation into the routine clinical practice where it will be applied during the next years in order to obtain further evidence regarding the usefulness of this new tool in the transplant settings.

Part VI

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REFERENCES

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Part VII

ANNEXES

ELSEVIER

Monitoring Cytomegalovirus T-Cell Immunity in Small Bowel/Multivisceral Transplant Recipients

A. Chiereghin, L. Gabrielli, C. Zanfi, E. Petrisli, A. Lauro, G. Piccirilli, F. Baccolini, A. Dazzi, M. Cescon, M.C. Morelli, A.D. Pinna, M.P. Landini, and T. Lazzarotto

ABSTRACT

Background. Cytomegalovirus (CMV) is a major cause of graft failure and posttransplantation mortality in intestinal/multivisceral transplantation. CMV infection exhibits a wide range of clinical manifestations from asymptomatic infection to severe CMV disease.

Study's Purpose. The purposes of this study were to assess the utility of measuring CMV-specific cellular immunity in bowel/multivisceral transplant recipients and to provide additional information on the risk of infection and development of CMV disease.

Methods. We studied 10 bowel/multivisceral transplant recipients to investigate the kinetics of CMV infection using real-time polymerase chain reaction (on blood and biopsy tissue samples) and CMV-specific T-cell reconstitution by Enzyme-linked ImmunoSPOT Assay (ELISPOT) that enumerates Interferon-γ–secreting CMV-specific T cells upon in vitro stimulation with viral antigens (pp65 and IE-1).

Results. All patients were seropositive for CMV. According to the pattern of T-cell reconstitution occurring either within the first month after transplantation or later, patients were classified as early (n = 7) or late responders (n = 3). Clinically, early responder patients (3/7; 43%) experienced asymptomatic or mild CMV infections, whereas all late responders (3/3; 100%) developed moderate or severe CMV disease. A reduction in mean and peak CMV viral load was observed in early responders, whereas the onset time of infection did not differ significantly between early and late CMV responders.

Conclusions. A good and early reconstitution of CMV-specific T-cell immune responses after transplantation is a critical determinant in controlling CMV infections. Simultaneous monitoring of CMV infection and CMV-specific T-cell immunity predicts T-cell–mediated control of CMV infection.

HUMAN cytomegalovirus (CMV), a member of the Betaherpesvirinae, is one of the most common opportunistic pathogens complicating the care of transplant recipients, and a major cause of posttransplantation morbidity and mortality.[1] CMV infections in solid-organ transplant recipients give rise to a broad spectrum of clinical manifestations from asymptomatic infection to severe disease.[2] Prevention strategies have included preemptive and prophylactic approaches that have significantly decreased the burden of CMV disease following organ transplantation.[3]

CMV replication is largely controlled by cellular immunity.[4] Over the past decade, conceptual and technical advances have substantially improved T-cell assays; it is now

From the Operative Unit of Clinical Microbiology, Laboratory of Virology (A.C., L.G., E.P., G.P., F.B., M.P.L., T.L.), and Department of Surgery and Transplantation (C.Z., A.L., A.D., M.C., M.C.M., A.D.P.), St. Orsola Malpighi General Hospital, University of Bologna, Bologna, Italy.

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Address reprint requests to Tiziana Lazzarotto, PhD, Operative Unit of Clinical Microbiology, St. Orsola Malpighi General Hospital, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy. E-mail: tiziana.lazzarotto@aosp.bo.it

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possible to functionally characterize viral- specific T-cell immunity.[5] Several studies have analyzed the correlation between CMV-specific T-cell responses and CMV infections by evaluating CD4+ and CD8+ T cell immune responses in recipients of kidney, liver, lung, hematopoietic stem cell or heart transplants. The results have demonstrated the importance of CMV-specific CD4+ and CD8+ T-cell responses in the control of CMV infections after transplantation.[3,6–19] The aim of our study was to assess the utility of monitoring CMV-specific cellular immunity in bowel/multivisceral transplant recipients to evaluate the risk of the development of severe CMV infection.

METHODS

Selection and Description of Participants

From March 2005 to January 2009, we enrolled 10 patients undergoing intestinal small bowel/multivisceral transplantation. The study population comprised recipients of 8 isolated intestinal and 2 multivisceral transplants (without liver). All patients were adults, with a male/female ratio of 7:3 and a mean age of 34 years. All transplant recipients and their respective donors were seropositive for CMV at the time of transplantation (D+/R+).

Technical Information

All patients received prophylaxis with intravenous ganciclovir (5 mg/kg per dose twice a day) and intravenous CMV immune globulin (3500 U every other day) for 2 weeks, followed by oral valganciclovir (450 mg/d) until the twelfth month after transplantation.

The immunosuppressive regimen was based on induction therapy with alemtuzumab (Campath, Genzyme Europe BV-NL) and low-dose tacrolimus (Prograf, Astellas Pharma US, Deerfield, Ill, USA) maintenance treatment. Alemtuzumab was administered in 2 doses of 0.3 mg/kg after graft reperfusion and on day 3. Tacrolimus, initially intravenous then oral, was administered at a dose of 0.075 mg/kg twice daily with target tacrolimus trough blood levels of 8–12 ng/mL. Steroids were added in cases of acute cellular rejection episodes.

Routine follow-up of CMV infection was performed on blood samples using CMV pp65 antigenemia assay as previously described in detail[20] and on intestinal biopsy specimens using real-time polymerase chain reaction (PCR; Q-CMV Real Time Complete Kit, Nanogen Advanced Diagnostics SRL, Turin Italy).

Blood samples were collected every week during the first 3 months, every 2 weeks until the sixth month, and monthly from then onward. Intestinal biopsies were performed during routine ileoscopy to check rejection status. Additional blood and biopsy samples were taken if clinically indicated. Pre-emptive CMV therapy was started when the antigenemia count was >1 positive cell/200,000 polymorphonuclear leukocytes in peripheral blood (PMNLs) or when a positive CMV tissue load was >100 copies/μg DNA. The findings were correlated with suspicious histology and/or clinical indications. Retrospectively, we performed virological and immunological monitoring on blood samples collected during the first 4 months posttransplantation. Virological surveillance was performed using CMV quantitative real-time PCR (Q-CMV Real Time Complete Kit, Nanogen Advanced Diagnostics SRL) at the time of transplantation and then weekly; we examined 140 blood samples. The immunological surveillance was carried out using Enzyme-linked ImmunoSPOT assay (ELISPOT; EliSpot Interferon-γ Basis Kit; Nanogen Advanced Diagnostics SRL) on blood samples collected at the time of transplantation and then monthly; we examined 50 samples.

CMV Quantitative Real-Time PCR

DNA was extracted from 100 μL of ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood using the NucliSens easyMAG System (bioMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. An aliquot of 5 μL of these extracted DNA samples was used for the CMV quantitative real-time PCR on the ABI Prism 7300 Real Time PCR System (PE Applied Biosystem, Foster City, Calif, United States). Primers and probes are located in the highly conserved major immediate early antigen (MIEA) region of the CMV genome. The limit of assay detection was 500 copies/mL of blood; CMV load was reported as number of copies/mL.

ELISPOT Assay

This assay enumerates interferon-γ-secreting CMV-specific T cells upon in vitro stimulation with viral antigens. ELISPOT assays provided CMV-specific quantitation of both CD4+ and CD8+ cells. Blood samples were collected in sodium citrate–treated tubes. Peripheral blood leukocites (PBLs) were isolated by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). An aliquot of 2×10^5 T cells were incubated in the anti–INF-γ antibody coated wells together with CMV-specific antigens (IE1 and pp65-UL83) for 20–24 hours in a CO_2 incubator. In response to the antigen, T cells specifically release INF-γ, which is then bound by the antibody coated to the well. Cytokine release is visualized by an enzyme-labeled detection antibody. The end results are blue spots, each of which represents the area of one cell secreting several molecules of INF-γ.[21] Colored spots were counted using a computer-assisted image analysis system (AID EliSpot Reader System, Strassberg, Germany). Negative and positive controls were included to check each sample as well as the whole assay. Cells incubated with medium alone and with phytohemagglutinin (PHA-P) mitogen were used as negative and positive controls, respectively.

A response was considered positive when the number of spot-forming cells (SFCs) in the wells stimulated with peptides was $\geq$5. The CMV T-cell immune response was considered weak when the number of SFCs was between 5 and 20, good between 20 and 100, and very good >100. The assay could only be performed if there was a sufficient quantity of isolated lymphocytes (2×10^6 cells) in the collected blood (20 mL).

Statistical Techniques

Two-group comparisons of mean values were statistically assessed using Student t test for independent samples. $P < .05$ was considered significant.

RESULTS

According to the pattern of T-cell reconstitution posttransplantation, there were 10 recipients classified as having either an early or a late CMV-specific T-cell response. Early and late CMV responders showed reconstitution of CMV-specific T-cell responses within and after 1 month posttransplantation, respectively. Seven of the 10 transplant recipients (70%) displayed early CMV-specific T-cell responses,

Table 1. Association of Early Vs. Late CMV-Specific T-Cell Response With Infection and Onset of Infection

T-Cell Response	No. of Patients	No. of CMV-Infected Patients (percentage)	Onset of Infection Days Posttransplantation Mean
Early	7	3 (43%)	82
Late	3	3 (100%)	62
Total	10	6	
P			0.549* (−64.99–104.99)†

*Student test.
†95% confidence interval of the difference between the means.

whereas 3 (30%) had late CMV-specific T-cell responses, as detected using the ELISPOT assay (Table 1).

The virological tests showed CMV infections in 6 of the 10 recipients (60%). Specifically, 3 of the 7 early CMV responders (43%) developed CMV infections; whereas all (3/3; 100%) late CMV-specific response patients developed CMV infections (Table 1).

Clinically, 2 of the 3 infected patients in the group of early CMV responders were asymptomatic and 1 suffered from a mild CMV infection (fever). All late CMV responders developed symptomatic CMV infections: 1 mild (fever) and 2 severe. One of these 2 patients underwent graftectomy for CMV enteritis with rejection. The patient died due to sepsis on day 69 posttransplantation. The other patient with severe symptomatic infection developed CMV pneumonia and died on day 125 after transplantation from sepsis.

The early responder patient with CMV infection died due to fungal sepsis at 150 days after transplantation. (The fungus was not identified.) The mortality rate among CMV-infected patients was 50% (3/6 patients) and the causes of death were sepsis in all cases. No patients without CMV infection died.

Focusing our attention on patients with CMV infection we observed that the time to when CMV DNA was first detected after transplantation did not differ significantly between early and late responders (mean, 82 days vs 62 days, respectively; Table 1). In contrast, mean and peak levels of CMV DNA in blood were significantly higher for late than for early responders (Table 2).

Figure 1 shows a patient with good reconstitution of CMV-specific T-cell responses in the first month posttransplantation (early CMV responder). The CMV-specific T-cell response was also good/very good over the following 4 months. Monitoring of CMV infection in the first 4 months posttransplantation disclosed no infection (viral load in the blood was < 500 copies/mL). This patient has had a good clinical outcome to date (3 years posttransplantation).

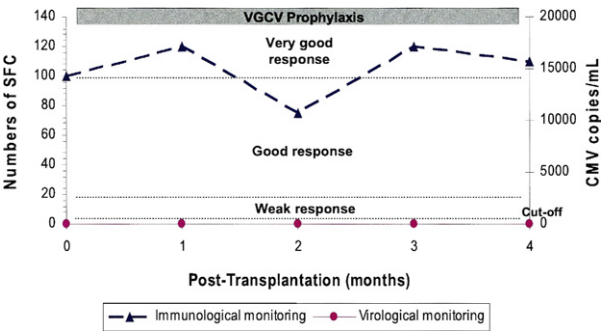

Fig 1. Early CMV responder. Kinetics of CMV-specific T-cell reconstitution and CMV infection during the first 4 months posttransplantation. Abbrevations: VGCV, valganciclovir; SFC, spot-forming cells.

Figure 2 reports an example of a patient who did not reconstitute CMV-specific T-cell responses within the first month after transplantation (as late CMV responder). A weak response ($5 \leq SFCs \leq 20$) was also present over the following weeks. At 3 months posttransplantation the patient developed a CMV infection with a high viral load in the blood (peak viral load, 152,300 copies/mL), although the patient was receiving prophylaxis treatment. Clinically, this patient experienced a severe symptomatic CMV infection presenting as CMV pneumonia, which was unsuccessfully treated with intravenous ganciclovir, and died due to sepsis on day 125 posttransplantation.

DISCUSSION

By virtue of its unique immunological, physiological, and bacteriological nature, the intestine remains the most difficult abdominal organ to transplant.[22] The vigorous rejection caused by the particularly high immunogenicity of the lymphocyte-rich intestinal allograft and the unique capacity of the intestine to mount a graft-versus-host disease (GVHD) demand greater degrees of immunosuppression in intestinal than recipients of other transplanted organs.[22,23] Advances in immunosuppressive therapy have lengthened the survival of intestinal transplant recipients, but the strong immunosuppressive protocols have been associated with an increased risk of developing tissue-invasive infections.[24] Among viral infections, CMV is a major cause of graft failure and posttransplantation mor-

Table 2. Association of Early Vs. Late CMV-Specific T-Cell Response With Viral Blood Load in the First 4 Months Posttransplantation

T-Cell Response	No. of Patients with CMV Infection	Viral Blood Load Copies/mL Mean	Peak Viral Blood Load Copies/mL Mean
Early	3	2,500	4,900
Late	3	37,000	118,000
Total	6		
P		0.052* (−69324–324)†	0.029* (−207676–18523)†

*Student's test.
†95% confidence interval of the difference between the means.

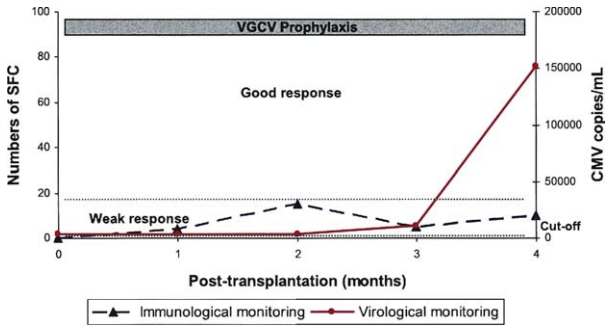

Fig 2. Late CMV responder. Kinetics of CMV-specific T-cell reconstitution and CMV infection during the first 4 months posttransplantation. Abbrevations: VGCV, valganciclovir; SFC, spot-forming cells.

tality in intestinal/multivisceral transplantation, playing a major role in the first 6 months after transplantation.[25,26]

We monitored 10 small bowel/multivisceral transplant recipients, observing that the total prevalence of CMV infection in the first 4 months after transplantation was 60% despite prophylactic therapy. All of these patients were CMV-seropositive. Studying the correlation between CMV-specific T-cell responses and CMV infections, we observed that CMV infections occurred in 3 of the 7 early CMV responders (43%) and in all 3 late CMV responders (100%). Moreover, moderate and severe CMV infections were strongly associated with late CMV responders (1/3 and 2/3 patients, respectively); however, early responders developed asymptomatic (2/3 patients) or mild (1/3 patients) CMV infections. Therefore, a CMV-specific T-cell immune response in the first month after transplantation was associated with a lower rate and lesser severity of CMV infection. In addition, the CMV-specific T-cell immune response in the first month after transplantation was associated with a reduced mean and peak CMV viral load in the blood. This observation suggests that early reconstitution of CMV-specific cellular immunity posttransplantation is a critical determinant controlling CMV infection, although the onset of infection did not differ significantly between early and late CMV responders.

In addition, all of our transplant recipients received prolonged valgancyclovir prophylaxis. Only 3 of 10 patients failed to reconstitute CMV T-cell responses within the early period, which may suggest that the prolonged prophylaxis did not influence the evolution of the T-cell response.

As already implemented in other organ transplants, we believe that immunologic monitoring in addition to virological monitoring is needed in the highly susceptible intestinal transplant recipients to ensure the best management of CMV infection. Immunological surveillance after transplantation may serve to select a subgroup of patients at high-risk of CMV-related disease requiring closer viral monitoring and more careful follow-up. Because prolonged prophylaxis increases the cost of drugs and may give rise to antiviral resistance and toxicity, treatment could be tailored to the individual patient, maintaining prophylaxis in high-risk patients and suspending therapy earlier in those at lowest risk. Additional prospective studies are required to guide safe changes in anti-CMV prophylaxis.

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Immunosuppression Modifications Based on an Immune Response Assay: Results of a Randomized, Controlled Trial

Matteo Ravaioli, MD,[1] Flavia Neri, MD,[2] Tiziana Lazzarotto, MD, PhD,[3] Valentina Rosa Bertuzzo, MD,[2] Paolo Di Gioia, MD, PhD,[2] Giacomo Stacchini, MD,[2] Maria Cristina Morelli, MD,[2] Giorgio Ercolani, MD, PhD,[2] Matteo Cescon, MD, PhD,[2] Angela Chiereghin, PhD,[3] Massimo Del Gaudio, MD, PhD,[2] Alessandro Cucchetti, MD, PhD,[2] and Antonio D. Pinna, MD, PhD[2]

Background. An immune function assay shows promise for identifying solid organ recipients at risk for infection or rejection. The following randomized prospective study was designed to assess the clinical benefits of adjusting immunosuppressive therapy in liver recipients based on immune function assay results. **Methods.** Adult liver recipients were randomized to standard practice (control group; n = 102) or serial immune function testing (interventional group; n = 100) performed with a commercially available in vitro diagnostic assay (ImmuKnow; Viracor-IBT Laboratories, Lee's Summit, MO) before transplantation, immediately after surgery and at day 1, weeks 1 to 4, 6, and 8, and months 3 to 6, 9, and 12. The assay was repeated within 7 days of suspected/confirmed rejection/infection and within 1 week after event resolution. **Results.** Based on immune function values, tacrolimus doses were reduced 25% when values were less than 130 ng/mL adenosine triphosphate (low immune cell response) and increased 25% when values were greater than 450 ng/mL adenosine triphosphate (strong immune cell response). The 1-year patient survival was significantly higher in the interventional arm (95% vs 82%; $P < 0.01$) and the incidence of infections longer than 14 days after transplantation was significantly lower among patients in the interventional arm (42.0% vs. 54.9%, $P < 0.05$). The difference in infection rates was because of lower bacterial (32% vs 46%; $P < 0.05$) and fungal infection (2% vs 11%; $P < 0.05$). Among recipients without adverse events, the study group had lower tacrolimus dosages and blood levels. **Conclusions.** Immune function testing provided additional data which helped optimize immunosuppression and improve patient outcomes.

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Most solid organ transplant recipients require lifelong treatment with potent immunosuppressant medications which reduce the risk of allograft rejection but increase patient morbidity and mortality after long-term use. Commonly reported adverse events associated with immunosuppressive therapy include hypertension (77%), hyperlipidemia (66%), renal toxicity (50%), obesity (40%), cancer (26%), and diabetes mellitus (22%).[1] During the immediate postoperative period, overimmunosuppression is associated with a greater incidence of infection and sepsis. Optimal immunosuppressive therapy balances the risk of rejection caused by an inadequately suppressed immune system and the risk of infection, cancer, and drug toxicity caused by overimmunosuppression. Drug therapy must be carefully tailored to each transplant recipient because of the differences in race, sex, metabolism, multiple drug regimens, and type of allograft transplant.

An immune function assay has been cleared by the U.S. Food and Drug Administration for measuring changes in cell-mediated immunity in solid organ transplant recipients undergoing immunosuppressive therapy (ImmuKnow; Viracor-IBT Laboratories, Lee's Summit, MO).[2] Numerous retrospective and prospective studies have demonstrated the ability of this immune function assay—when used with

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[1] Sant'Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy.

[2] Department of General Surgery and Transplantation, Sant'Orsola-Malpighi Hospital, Bologna, Italy.

[3] Clinical Unit of Microbiology, University of Bologna, Bologna, Italy.

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M.R. participated in research design, data analysis, and writing the paper. F.N. participated in writing the paper. T.L. participated in performing the study and contributed new reagents and analytic tools. V.B. participated in data analysis. P. D.G. participated in data collection and analysis. S.G. participated in data collection and analysis. M.C.M. participated in performing the study. G.E. participated in performing the study. M.C. participated in performing the study. A. C. participated in performing the study and contributed new reagents and analytic tools. M.D.G. participated in performing the study. A.C. participated in performing the study. A.D.P. participated in research design and performing the study.

Correspondence: Matteo Ravaioli, MD, Department of General Surgery and Transplantation, Sant'Orsola-Malpighi Hospital, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy. (mrava1@hotmail.com; matteo.ravaioli@aosp.bo.it)

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other biomarkers—to identify patients at risk of organ rejection[3–7] and infection[5,8–15] across a range of organs. The assay can also distinguish between rejection and recurrent hepatitis C infection,[16] facilitate conversion from one immunosuppressant to another,[17,18] and may be useful for identifying patients with increased risk of short-term mortality,[19] predicting posttransplant recurrence of hepatocellular carcinoma (HCC)[12] and evaluating immune status of patients with de novo malignancy.[20]

Previously, the role of this immune function test was retrospectively assessed for monitoring and adjustment of immunosuppression in patients after orthotopic liver transplant.[21] Based on those promising results, a prospective randomized study was designed to assess the clinical benefit of adjusting immunosuppressive therapy based on immune function assay results compared to a group managed with the standard clinical practice. The outcome data of this analysis are reported here.

MATERIALS AND METHODS

Study Design

This was a prospective, randomized, parallel, blinded, interventional trial comparing the outcomes of adult liver transplant recipients whose immunosuppressive therapy is managed by previous standard practice (control group) or by adjusting therapy based on cell-mediated immune responses determined by the immune function assay (interventional group). Outcomes were assessed 12 months after transplantation among liver recipients enrolled between July 2008 and March 2013. This study was approved by the local hospital ethics committee.

Patients

Consecutive adult liver transplant recipients not participating in other studies and who provided informed consent were enrolled. There were no exclusions pertaining to the cause of end-stage liver disease (ESLD). To ensure treatment groups were balanced, enrolled patients were randomized 1:1 based on the cause of ESLD, model for end-stage liver disease (MELD) score, the presence of HCC, and sex. A significant number of liver recipients with ESLD secondary to hepatitis C virus (HCV) infection were not included because of a parallel study.

Immune Function Testing

Immune function testing was performed with a commercially available in vitro diagnostic assay cleared by the U.S. Food and Drug Administration that uses whole blood samples collected in sodium heparin vacuum tubes (ImmuKnow; Cylex, Inc., now part of ViraCor-IBT Laboratories, Inc., Lee's Summit, MO). The assay is designed to detect cell-mediated immunity by measuring the concentration of ATP from CD4+ cells after stimulation and was performed as directed by the manufacturer.[2] Values of 225 ng/mL or lower ATP suggest a low immune cell response and values of 525 ng/mL or higher ATP suggest a strong immune cell response.[22]

To prospectively evaluate the management of immunosuppression based on their immune function assay values, all patients were tested with the assay before liver transplantation, immediately after surgery, and at each clinic visit occurring at approximately day 1, weeks 1 to 4, 6 and 8, and months 3 to 6, 9, and 12.[2] The assay was repeated within 7 days of a suspected or confirmed rejection or infection and again within 1 week after resolution of the event.

Intervention

Immunosuppressive therapy consisted of tacrolimus and a steroid taper according to standard practice at our center. The steroid tapering was planned as follows: 1 g methylprednisolone before liver reperfusion, 200 mg at day 1, 160 mg at day 2, 120 mg at day 3, 80 mg at day 4, and 40 mg at day 5. Subsequently, oral prednisone 25 mg was administered daily and gradually stopped during the next 6 months.

The target tacrolimus plasma levels were 8 to 12 ng/mL at 4 months and 6 to 10 ng/mL after 4 months.[23] Steroids were not tapered in patients transplanted for primary biliary cirrhosis, autoimmune cirrhosis, or primary sclerosing cholangitis. In cases of renal impairment (defined as plasma creatinine > 1.5 mg/dL), tacrolimus levels were reduced to 4 to 6 ng/mL and mycophenolic acid or a mechanistic target of rapamycin (mTOR) inhibitor was added.

Based on immune function assay values and our clinical experience, the following was used to adjust therapy in the Interventional group: the tacrolimus dose was reduced by 25% when immune function values were less than 130 ng/mL ATP (low immune response) and increased by 25% when assay values were greater than 450 ng/mL ATP (strong immune response). Tacrolimus dosage changes were made until the assay values stabilized between 130 and 450 ng/mL ATP. These values were previously documented as thresholds for risks of infection and rejection, respectively, with a value of 280 ng/mL corresponding with the greatest negative predictive value for either event.[7] Treating physicians in the control group were blinded to the immune assay results, and immunosuppression was adjusted according to standard practice.

Adverse Events

Rejection was diagnosed by biopsy and graded as indeterminate, mild, moderate, or severe according to the Banff schema.[24] Infection was defined as a microbial phenomenon characterized by an inflammatory response (pain, heat, redness, swelling)[25] to the presence of microorganisms and were classified according to standardized criteria.[26] In this sense, bacterial, viral, parasitic, and mycotic infections were included.

Systemic inflammatory response syndrome was defined as a systemic inflammatory response to a variety of severe clinical insults, and it was considered to be manifested by the presence of 2 or more of the following conditions: (1) body temperature greater than 38 °C or less than 36 °C; (2) heart rate greater than 90 beats/min; (3) respiratory rate greater than 20/min or $PaCO_2$ less than 32 mm Hg; (4) white blood count greater than 12,000/mm^3 or less than 4000/mm^3. Sepsis was defined as a systemic response to infection manifested by systemic inflammatory response syndrome,[27] and it was deemed severe in presence of organ dysfunction or when requiring hospitalization. Cytomegalovirus (CMV) viral syndrome was defined when CMV-DNA or CMV-antigen positivity was associated with fever greater than 38°C for 2 days of unexplained origin, leucopenia, myalgia, or arthralgia.[28]

Infectious Screening Protocol

The CMV-DNA was routinely measured once weekly during the hospital stay, as well as hepatitis B surface antigen and hepatitis B surface antibody in patients who underwent liver

transplantation for hepatitis B virus (HBV) infection or who received a graft from a HBcAb donor. After discharge, patients were followed in a dedicated outpatient clinic. Complete blood count, renal and hepatic function, urinalysis, and CMV-DNA were measured at every visit. Hepatitis B surface antigen, hepatitis B surface antibody, HBV-DNA, and HCV-RNA were also measured if clinically indicated (ie, HBV-positive patients, HCV-positive patients, and recipients of HBcAb-positive graft). Blood, urine, ascites, and bronchoalveolar cultures were performed any time there was clinical suspicion of infection but surveillance cultures were not routinely performed. More specific microbiological tests, such as galactomannan Ag determination and interferon- γ release assay for latent tuberculosis infection, were performed as second-line tests. Chest roentgenograms were performed any time there was the clinical suspicion of pulmonary infection. Computed tomography (CT) was the second-line test if the roentgenogram was dubious. Abdominal ultrasound or CT scan was performed any time there was a clinical suspicion of intra-abdominal infections and to rule out arterial/biliary graft complications.

Infection Prophylaxis Protocol

The following medications were administrated as infection prophylaxis:

- Trimethoprim/sulfamethoxazole tablets 60 mg/800 mg, 3 times weekly for 12 months after liver transplantation as *Pneumocystis carinii* prophylaxis,
- Nystatin oral suspension 100,000 IU/mL, 5 mL 4 times daily until steroid treatment interruption as candidiasis prophylaxis,
- Intravenous ganciclovir at a dose based on creatinine clearance until postoperative day 7 in cases of donor/recipient CMV serology mismatch (donor IgG+/recipient IgG–); subsequently, oral valganciclovir at a dose based on creatinine clearance for 12 months after liver transplantation. No CMV infection prophylaxis is routinely administered to IgG+ recipients.

Infection Treatments

An empiric antimicrobial therapy was started in case of clinical suspicion of infection. Blood and urine cultures were performed when clinically indicated and ascites and bronchoalveolar lavage cultures were performed when available. More specific microbiological tests were performed as second line tests (galactomannan Ag culture; QuantiFERON, Cellestis LTD, Chadstone, Australia). Radiological tests (chest X-ray, abdominal CT scan) were used to identify infection sites. If the cultures were positive, the antimicrobial treatment was modified according to the antibiograms.

Outcomes

The primary study outcome was a comparison of patient survival, infections, allograft rejection, and graft loss between the control and interventional groups. Secondary outcomes were differences in the dose of primary immunosuppressants among patients without adverse events, posttransplant renal failure (considering dialysis, plasma creatinine level and glomerular filtration rate according to the formula: GFR (mL/min/1.73 m^2) = 175 × $(S_{cr})^{-1.154}$ × $(Age)^{-0.203}$ × (0.742 if female) × (1.212 if African American), diabetes (treatment with insulin or oral antidiabetic agents), HCC recurrence, and the addition of adjunctive immunosuppressants, such as an antiproliferative, mTOR, or steroid.

Statistical Analysis

Published data of a 20% incidence of rejection[29,30] and 30% to 65% incidence of bacterial and CMV infections[31,32] were used to calculate the required number of enrolled patients. We hypothesized that immune function testing may lower the risk of these events by 30%. Using Fisher exact test with an α of 0.05 and β of 0.80, a sample size of 103 patients was required for each group. Univariate analysis was performed using Student *t* test for continuous variables and Fisher exact test for categorical variables. For 2-tailed calculations, differences were considered significant for *P* less than 0.05.

The patient survival after liver transplantation was calculated by the Kaplan-Meier method starting from the day of LT to the day of death or to the most recent follow-up visit. Differences were compared by the log-rank test, and variables were evaluated in the multivariate analysis using Cox proportional hazard model (Figure 1).

Ethics

The study was conducted in accordance with the declaration of Helsinki and followed the Good Clinical Practice guidelines of the International Conference on Harmonization. The protocol used in this study was approved by the Independent Ethics Committee at Sant'Orsola-Malpighi Hospital of the Università di Bologna. ClinicalTrials.gov Identifier: NCT01764581.

RESULTS

Patient Characteristics and Allocation

A total of 202 de novo liver transplant patients 18 years or older received a deceased or extended criteria donor liver between July 2008 and March 2013. Patients were randomized 1:1 to the control (standard practice) group (n = 102) and the interventional (immune function testing) group (n = 100). There were no statistical differences between the groups with respect to group allocation, posttransplantation follow-up, and analysis.

The demographics, cause of ESLD, and surgical procedures of the enrolled patients are summarized in Table 1. There were no significant differences with respect to age, sex, race, MELD score, donor MELD (D-MELD) score,[33] or reason for liver transplantation. All donors were deceased, and no differences were seen between standard or extended criteria donors.

Notably, there was an inverse correlation between immune function assay values and MELD scores prior to transplantation. Patients with MELD scores greater than 20 had statistically lower immune function values than patients with lower MELD scores (median 58 ng/mL adenosine triphosphate [ATP] vs. 114 ng/mL ATP; $P < 0.05$).

Patient Survival

The actuarial survival rate of patients was 89% in the interventional group compared to 78.4% in the control group ($P < 0.05$) and 1-year patient survival was significantly higher in the interventional group (95% vs 82%, $P < 0.01$), as reported in Figure 2.

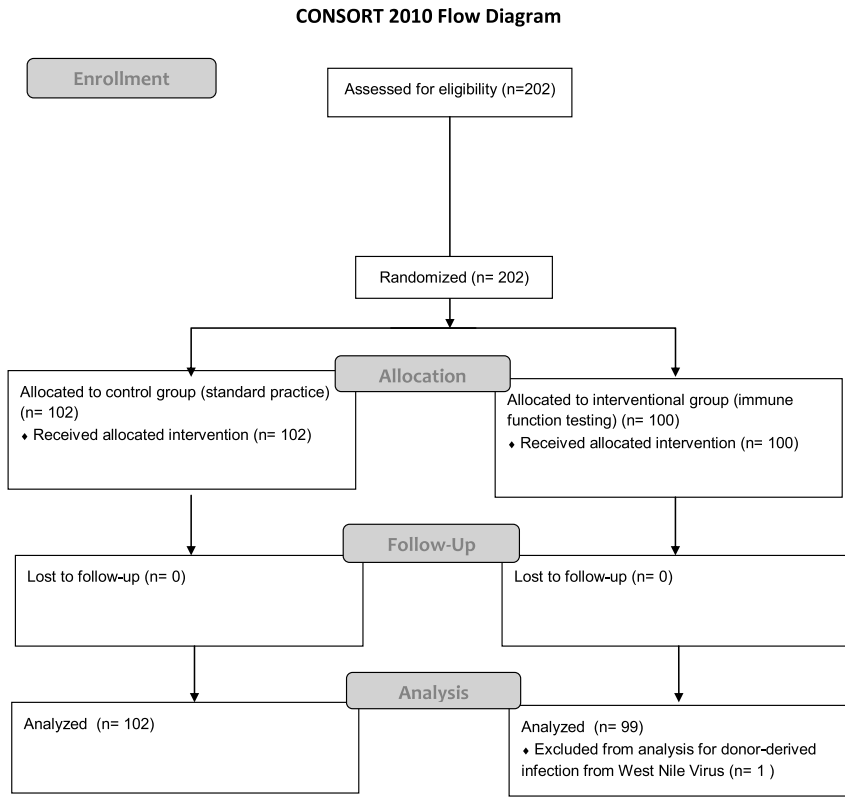

FIGURE 1.

Among the 33 patients who died, 11 were in the interventional group (33%) and 22 were in the control group (67%). Eleven patients died from infections (33.3%), 9 patients from multiorgan failure (27.3%), 6 from recurrent HCC or de novo tumor (18.2%), 4 from surgical complication (12.1%), and 3 from recurrent HCV hepatitis (9.1%).

One patient in the control group and 2 patients in the interventional group were retransplanted because primary graft nonfunction.

The univariate analysis showed a statistical correlation with a lower patient survival at 1 year for the cases with D-MELD greater than 1600 (81.5% vs. 91%, $P < 0.05$) and with infection events (82.6% vs 96%, $P < 0.01$), whereas the presence of HCC or HCV-positive, acute rejection, or other recipient and donor features did not demonstrate any correlation with the patient survival.

The multivariate analysis, including the D-MELD greater than 1600 and the study groups, showed that the interventional arm was independently associated with a higher 1-year patient survival ($P < 0.01$; 95% confidence interval, 1.3–9.5).

Adverse Events

The numbers of adverse events in the 2 study groups are shown in Table 2. There was no statistical difference between cohorts with respect to acute rejection events, which were mild in most cases according to Banff scores with no occurrence of steroid-resistant or chronic rejection.

The infection events after the first 2 weeks posttransplant were significantly lower in the interventional group (42% vs. 54.9%, $P < 0.05$) corresponding with improved patient

TABLE 1.

Demographics and clinical features of the study populations

	All patients (N = 202)	Intervention group (n = 100)	Control group (n = 102)	P
Recipients				
Mean age (SD), y	54 (11)	55 (11)	53 (12)	n.s.
Male sex, n (%)	127 (62.9)	66 (66)	61 (59.8)	n.s.
Viral hepatitis, n (%)				
HCV-positive	53 (26.2)	25 (25)	28 (27.5)	n.s.
HBV-positive	81 (40)	39 (39)	42 (41.2)	n.s.
Virus negative	68 (33.7)	36 (36)	32 (31.4)	n.s.
HCC, n (%)	70 (34.7)	32 (33)[a]	38 (37.3)[b]	n.s.
Mean MELD score (SD)	20 (10)	19 (9)	21 (10)	n.s.
MELD > 20, n (%)[c]	99 (49)	44 (44)	55 (53.9)	n.s.
Mean BMI (SD), kg/m^2	25.5 (4.6)	25.4 (4.5)	25.5 (4.7)	n.s.
Donors				
Mean age (SD), y	54.54 (20.3)	53.22 (19.3)	55.7 (21.3)	n.s.
Male sex, n (%)	106 (52.5)	57 (57)	49 (48)	n.s.
Cause of death, n (%)				
Cerebrovascular accident	132 (65.3)	63 (63)	69 (67.6)	n.s.
Trauma	39 (19.3)	21 (21)	18 (17.6)	n.s.
Other	31 (15.3)	16 (16)	15 (14.7)	n.s.
Mean BMI (SD), kg/m^2	26.1 (5.2)	25.9 (5.1)	26.5 (5.3)	n.s.
Mean ischemia time (SD), min	396 (102)	400 (127)	383 (69)	n.s.
D-MELD score (SD)	1162 (708)	1090 (705)	1234 (706)	n.s.
D-MELD >1600, n (%)	54 (27.1)	22 (22.2)	32 (32)	n.s.

[a] (T1 = 13, T2 = 11; T3 = 8).
[b] (T1 = 17, T2 = 12, T3,=9).
[c] MELD score was measured 1 day before liver transplantation.
BMI, body mass index.

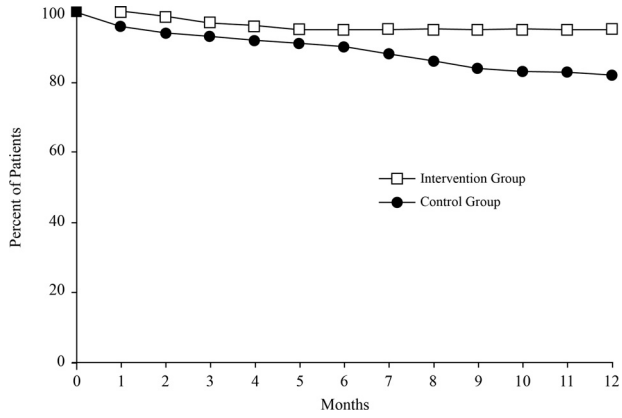

FIGURE 2. Patients survival 1 year after transplantation for intervention and control groups. The patient survival after liver transplantation was calculated by the Kaplan-Meier method starting from the day of LT to the day of death or to the most recent follow-up visit. The 1-year patient survival was significantly higher in the Interventional group (95% vs. 82%, $P < 0.01$).

survival in that group. This difference was further increased when selecting the recipients with MELD scores greater than 20 (Table 2).

Among the laboratory-confirmed infectious episodes that occurred after postoperative day 14, bacterial infections were responsible for most cases (57.1%), specifically, bacteremia (n = 45), pneumonia (n = 15), biliary tree infections (n = 6), urinary tract infections (n = 5), and ascites infection (n = 6). Viral infections were involved in 33.3% of cases including herpes simplex (n = 3), varicella zoster (n = 2), human herpes virus 6 (n = 1), human herpes virus 1 (n = 2), human herpes virus 8 (n = 2), West Nile virus (n = 1), and CMV (N = 33). Fungal infections were responsible for the remaining cases (9.6%): *Aspergillus* pneumonia (n = 3), cerebral *Aspergillosis* (n = 1), and candidemia (n = 9).

Overall, patients in the control group experienced a higher incidence of infections. Among patients with bacterial infections (N = 77; 57.1%), 47 (46.1%) occurred in the control group versus 32 (32%) in the interventional group ($P < 0.05$). Similarly, among patients with fungal infections (n = 13; 9.6%), 11 (10.8%) occurred on the control group versus 2 (2%) in the interventional group ($P < 0.05$). There was no significant difference in the number of patients with viral infections in the control and intervention groups (23 vs 22, respectively). Patients in the control group were twice as likely to be hospitalized for infections with longer stays compared to patients in the interventional group (relative risk = 2.1).

Immunosuppression Adjustment: Patient Example

An example case of immunosuppression adjustment based on immune function assay values and clinical condition is described in an immune monitoring profile. This 54-year-old man was transplanted in June 2009 for cryptogenic cirrhosis. His immunosuppressive therapy was managed according to immune function testing as described above. Immune function values increased from 23 to 137 ng/mL ATP during the first posttransplant week and by week 2 had reached 285 ng/mL ATP. During this time, his immunosuppression therapy followed routine practice as these values did not persist below 130 ng/mL or exceed 450 ng/mL ATP. His immune function assay values decreased to 32 ng/mL ATP between days 14 and 50 and his tacrolimus (Prograf; Astellas Pharma S.p.A., Carugate, IT) was reduced from 12 mg per day (6 mg twice per day) to 9 mg per day (5 mg in the morning, 4 mg in the evening). As a result, his immune function value increased to 166 ng/mL ATP at 3 months. His immune function assay value decreased to 106 ng/mL at his 6-month clinic visit, and his tacrolimus was further adjusted to 4 mg per day (2 mg twice per day). At 9 months after transplantation, he was diagnosed with chronic renal failure and at 12 months his immune function assay value was 65 ng/mL ATP. Tacrolimus was further reduced to 2 mg per day and then to 1 mg per day and mycophenolate mofetil (CellCept; Roche Registration Limited, Welwyn Garden City, UK) was added at a dose of 1000 mg per day (500 mg twice per day). No infections or rejections occurred during the course of this patient's first posttransplant year.

TABLE 2.

Comparison of outcomes at 12 months of follow-up

	All patients (N = 202)	Intervention group (n = 100)	Control group (n = 102)	*P*
Patient survival, %	170 (84.2)	89 (89.0)[a]	80 (78.4)[b]	<0.05
Event-free recipients, %	83 (58.9)	41 (41.0)	42 (41.2)	n.s.
Infectious episodes >14 d after transplantation, %	98 (48.5)	42 (42.0)	56 (54.9)	<0.05
Acute rejections, %	33 (16.3)	19 (19.0)	14 (13.7)	n.s.
Recipients with >3 infection, %	21 (10.4)	11 (11.0)	10 (9.8)	n.s.
Bacterial infections, %	77 (57.1)	32 (32.0)	47 (46.1)	<0.05
Fungal infections, %	13 (9.6)	2 (2.0)	11 (10.8)	<0.05
Viral infections, %	45 (33.3)	22 (22.0)	23 (22.5)	n.s.
	All patients, MELD >20 (N = 99)	Intervention group, MELD >20 (n = 44)	Control group, MELD >20 (n = 55)	
Patient survival, %	81 (81.8)	41 (93.2)	40 (72.7)	<0.01
Infectious episodes >14 d after transplantation, %	60 (60.6)	22 (50.0)	38 (69.1)	<0.05

[a] Cause of 11 deaths: multiple organ failure (n = 2, 18%), infections (n = 3, 27%), recurrent HCV hepatitis (n = 1, 9.1%), surgical complication (n = 2, 18%), and tumor-related causes (n = 3, 27%).
[b] Cause of 22 deaths: multiple organ failure (n = 7, 32%), infections (n = 8, 36%), recurrent HCV hepatitis (n = 2, 9.1%), technical reasons (n = 2, 9.1%), and tumor-related causes (n = 3; 14%). MELD scores were measured the day before liver transplantation.

Immune Function Assay, Tacrolimus Level, and Infections

Among recipients without adverse events during the first 3 months after transplantation, patients in the interventional group had significantly lower median tacrolimus doses (6 mg vs 8 mg, $P < 0.01$) and tacrolimus trough levels (8 mg/dL vs 9 mg/dL, $P < 0.01$). At 6 and 12 months after transplantation, the median tacrolimus level were 7 and 6 mg/dL, respectively, in the interventional group compared to 8 and 7 mg/dL in the control group (for each, $P < 0.05$).

At postoperative day 30, the number of infection events was statistically correlated with lower level of immune function assay values (median, 83 vs 178 ng/dL ATP; $P < 0.001$), as well as the tacrolimus daily dosage (median, 5 vs 4 mg/day, $P < 0.01$) and tacrolimus level (median, 7 vs 8 ng/mL; $P < 0.05$), which were lower in the infection group and therefore not effective to prevent them.

Dividing the patients according to the presence or the absence of infection, we found a correlation with a lower immune function assay at each timepoint during the 6-month posttransplant period, whereas the tacrolimus level showed a correlation only in the first month after transplantation (Figure 3).

The patients with rejection had a higher level of immune function assay values without reaching statistical difference (median, 211 vs 163 ng/dL; $P = 0.09$), while they had a significantly higher tacrolimus daily dosage and plasma level (median 8 vs 5 mg/day, $P < 0.001$ and median 9 vs 8 ng/mL; $P < 0.001$). Conversely, the tacrolimus dosage and levels were higher in the patients with rejection.

There were no between-group differences in the use of antiproliferatives (mycophenolic acid, mycophenolate mofetil, or azathioprine (GlaxoSmithKline S.p.A., Verona, IT), steroids or mTOR inhibitors.

DISCUSSION

The present randomized trial demonstrated the benefit of managing immunosuppression in adult liver transplant recipients using the ImmuKnow immune function assay. The ImmuKnow assay measures the ability of CD4+ cells to respond to mitogenic stimulation by phytohemagglutinin-L in vitro by quantifying the amount of ATP produced in CD4+ cells after stimulation.[22] The most commonly used immunosuppressive medications, such as cyclosporine and tacrolimus, inhibit lymphocytes and especially T lymphocytes such as CD4+ cells.[2] As the proliferation of CD8+ cells and other components of the cell-mediated immune response are largely under the control of CD4+ cells, the measurement of CD4+ activation is a more accurate reflection of global cell-mediated immune function. By combining sequential immune assay values with other routine tests, the treating transplant physician has a more complete immunological picture helpful in making more pondered decisions regarding therapy adjustments.

Similar to other reports, we found bacterial infections to be the most common type of infections[26]; however, using the immune function assay, the patients in the interventional arm of the study had a reduced number of bacterial and fungal infection events and improved the patient survival during the first year after transplantation.

These results are in agreement with other studies that demonstrated a correlation between low assay values and the incidence of recurrent hepatitis C viral infections,[7,10,21,29] human immunodeficiency virus/HCV-coinfected patients,[30] bacterial infections,[31] and invasive fungal infections[32] among liver transplant recipients. In 1 observational study, liver recipients with an immune function value less than 130 ng/mL ATP were 12 times more likely to develop an infection ($P < 0.001$),[9] and the results of a large meta-analysis concluded that the immune function test is a valid tool for determining the risk of further infection in adult liver transplant recipients.[8]

Similar to several previous studies, the immune function test values did not correlate with episodes of rejection in liver recipients[34] although some investigators have reported it to be useful for monitoring rejection.[6,31] The results of a recent study suggest the predictive power of the immune function assay may be improved by combining it with the CD4+ T lymphocyte count and CD4+/CD8+ ratio.[35]

The multivariate analysis including the variable D-MELD score, which summarizes the donor and recipient features, confirmed that the interventional arm had an independently higher survival (19% improvement) at the first year after transplantation compared to the control group with conventional postoperative management.

Early during the study, it was observed that patients with pretransplant MELD scores greater than 20 had significantly lower ImmuKnow values. This demonstrates that many patients are highly immunosuppressed before receiving any immunosuppressive therapy. As a result of this finding,

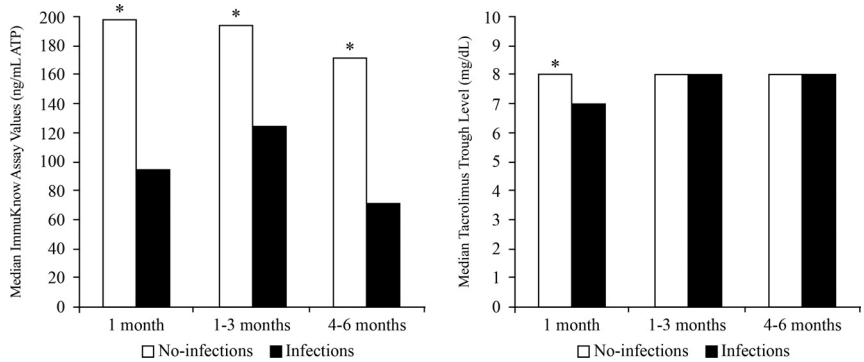

FIGURE 3. Plasma ImmuKnow and Tacrolimus values among with and without infections. Left, Median ImmuKnow assay values were significantly higher (Stronger immune response) among patients without infections at each time period after transplantation. *$P < 0.01$. Right, Median tacrolimus trough levels were significantly higher among patients without infections during the first month following transplantation. *$P < 0.01$.

we adjusted the amount of immunosuppression initially given to patients during transplant surgery by combining ImmuKnow values with MELD scores. These very sick patients, transplanted earlier with an allocation system based on MELD score,[36–38] are at higher risk of mortality due to infection,[39] demanding a strategy to reduce their immunosuppression.

Utilization of the ImmuKnow test results to optimize immunosuppression yielded improved patient survival and reduced infectious episodes in the interventional group as shown in Table 2. These findings were even more striking for the recipients with MELD scores greater than 20 in the interventional group who had a patient survival nearly 20% higher than that in the control group with the same MELD score.

Drug levels are often used to guide immunotherapy; however, this approach often results in drug toxicity, infection, or graft rejection.[40] We showed that the measurement of plasma tacrolimus levels is not sufficient to prevent infection as demonstrated in Figure 3. After the first month posttransplant, patients with or without infection had the same tacrolimus level, whereas the ImmuKnow values were significantly lower in the cases with infections. On the other hand, the immune assay should not be considered an alternative to the tacrolimus level, but it should be a piece of a complex puzzle of the immunosuppression of a transplant recipient. By using this assay, we were able to reduce the daily tacrolimus dosage and the trough level in the interventional group. This was demonstrated among the patients without adverse events because patients with infection were managed by reducing immunosuppression while increasing immunosuppression in patients with rejection. Therefore, if 1 group had higher rates of infection, an analysis of tacrolimus dose and plasma level would have suggested reducing immunosuppression in patients with infection.

The analysis of tacrolimus dosage and plasma levels was focused on recipients without adverse events to limit biases that may be caused from treating patients with various adverse events (increased immunosuppression for apparent rejections and decreased immunosuppression for diagnosed infections). The lower exposure to tacrolimus will likely improve the long-term renal function of patients when managed by ImmuKnow,[41] even if our data did not show any statistical significant difference of creatinine level and glomerular filtration rate at 6 and 12 month after transplantation (data not reported).

Because the use of steroids and other immunosuppressants were not subject to change based on ImmuKnow values, there were no differences in the amount of these agents used between the 2 groups.

There was higher patient survival among HCV-positive patients and those with HCC in the Interventional group (data not reported). These patients are supposed to benefit from lower immunosuppression to prevent viral and tumor recurrence of disease, but there were no statistical differences, and the number of cases was insufficient for any conclusive subanalysis.

It may be suggested that the same lower levels of immunosuppression among patients in the interventional group without adverse events can be achieved without using the ImmuKnow assay[42] and that the assay was less effective in preventing rejection events compared to infections. Performing another randomized study in which immunosuppression is minimized by using conventional criteria of clinical practice may provide additional data. Although the literature does not provide any suggestions for implementing this strategy,[47,48] the results of our study offers an effective tool to minimize immunosuppression, especially in patients with high MELD scores.

Our results showed a significant difference in patient survival and significantly lower bacterial and fungal infection rates between the study groups, which were otherwise comparable according to the donor and recipient features and the multivariate analysis confirmed this important data. Furthermore, the sample size and the statistical power of the study prevented any statistical bias.

In conclusion, the present prospective, interventional, controlled, and randomized study demonstrates that ImmuKnow provides a useful biomarker which enables optimizing immunosuppression to improve patient outcomes by preventing bacterial and fungal infections, reducing immunosuppressant drug use and improving 1-year patient survival after liver transplantation. These results are even more compelling in cases with MELD scores greater than 20.

ACKNOWLEDGMENTS

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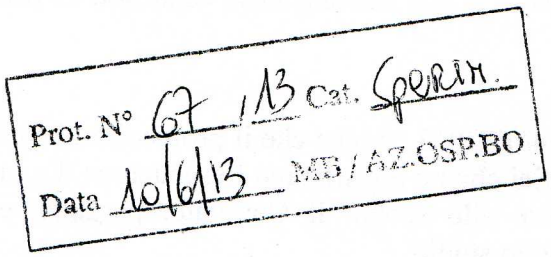
Comitato Etico Indipendente dell'Azienda Ospedaliero-Universitaria di Bologna, Policlinico S. Orsola-Malpighi

Bologna, lì 31 maggio 2013
Prot. nr. 1551/2013

Ns. riferimento da citare sempre nella corrispondenza:
n° 41/2013/U/Tess approvato il 14/05/2013

Chiar.ma Prof.ssa M.P. Landini
Direttore U.O. Microbiologia
Pad. 11 S. Orsola

E p.c.



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Investigational Drug Service
c/o U.O.Farmacia – Puggioli
Pad. 19 – S. Orsola
Policlinico S. Orsola-Malpighi

Gent.ma Dott.ssa Cristina Puggioli
Direttore U.O. Farmacia
Pad. 19 – S. Orsola
Policlinico S. Orsola-Malpighi

Oggetto: 41/2013/U/Tess – Studio monocentrico – Sperimentatore clinica "Disordini linfoproliferativi a cellule B associati all'infezione da Epstein-Barr virus nel paziente trapiantato di cellule staminali emopoietiche" – Prot. VIRO-13 – Studio promosso dal Dipartimento di Medicina Specialistica, Diagnostica e Sperimentale dell'Università degli studi di Bologna/U.O. Microbiologia in collaborazione con le UU.OO. Ematologia-Cavo f.f. e Pediatria-Pession
Sperimentatore Principale: Prof.ssa T. Lazzarotto
Direttore: Prof.ssa M.P. Landini

- Modulo di "Richiesta di parere al Comitato Etico per Sperimentazione con impiego di tessuti umani in vitro" sottoscritto dallo Sperimentatore Responsabile e dal Direttore dell'Unità Operativa, datato 17/04/2013
- Richiesta di autorizzazione all'Autorità Competente per la conduzione dello studio da parte del Promotore e Sperimentatore principale, datata 17/04/2013
- Protocollo di Studio, versione finale del 11/04/2013
- Sinossi del Protocollo, versione finale del 11/04/2013
- Informazioni per il paziente – Adulti, versione finale del 11/04/2013
- Consenso informato e consenso al trattamento dei dati sensibili - Adulti, versione finale del 11/04/2013
- Informazioni per il genitore del paziente, versione finale del 11/04/2013
- Consenso informato e consenso al trattamento dei dati sensibili - Minorenni, versione finale del 11/04/2013
- Lettera al Pediatra, versione del 11/04/2013
- Lettera al medico curante, versione del 11/04/2013
- CRF Adulti, versione finale del 11/04/2013
- CRF Minorenni, versione finale del 11/04/2013

Comitato Etico Indipendente dell'Azienda Ospedaliero-Universitaria di Bologna, Policlinico S. Orsola-Malpighi

- Dichiarazione di collaborazione allo Studio, sottoscritta dal Direttore dell'U.O. Pediatria il 17/04/2013
- Dichiarazione di collaborazione allo Studio, sottoscritta dal Direttore dell'U.O. Ematologia il 17/04/2013
- Dichiarazione di collaborazione allo Studio, sottoscritta dal Dott. D. Gibertoni del Dipartimento di Scienze Biomediche e Neuromotorie il 16/04/2013
- Dichiarazione sulla natura no profit dello studio e di non ricevere alcun compenso da parte dello Sperimentatore, datata 17/04/2013
- Curriculum vitae dello Sperimentatore

Il Comitato Etico, nella seduta del giorno 14/05/2013, ritiene che il protocollo di studio ed i suoi obiettivi soddisfacciano i criteri etici e scientifici che ne giustificano l'esecuzione. Le modalità del consenso informato sono corrette ed adeguate allo scopo. Il Comitato, pertanto, esprime all'unanimità **parere favorevole** alla conduzione dello studio.

Si precisa, tuttavia, che nelle informativa dovrà essere la frase "*I campioni di sangue necessari per tale test sono 4; 9 ml di sangue ciascuno e raccolti dopo 60, 180 e 360 giorni post-trapianto*" dal momento che non è specificata la seconda scadenza del prelievo (nel protocollo risulta "*Il monitoraggio immunologico verrà effettuato con il test ELISPOT su campioni di sangue periferico raccolto a 60, 100, 180 e 360 giorni post-trapianto*").

Si precisa, infine, che:

1. la documentazione modificata come sopra indicato dovrà essere inviata alla Segreteria del Comitato Etico che provvederà a verificarne la correttezza effettuando debita segnalazione entro 15 giorni dall'acquisizione dei documenti modificati esclusivamente nel caso in cui dall'esame dovessero emergere criticità; in assenza di riscontro entro tale termine i documenti sono da considerarsi approvati;
2. lo studio potrà essere avviato solo dopo aver ricevuto l'autorizzazione all'attivazione da parte della Direzione Generale dell'Azienda;
3. come previsto dalla normativa vigente in materia, lo Sperimentatore Responsabile dovrà comunicare al Comitato Etico le seguenti informazioni relative all'andamento dello studio: data di inizio arruolamento, data di fine arruolamento e data di conclusione dello studio. Inoltre, a partire dalla data di approvazione, dovrà essere fornito un rapporto annuale sullo stato di avanzamento dello studio. Per le suddette comunicazioni è possibile fare riferimento al modello disponibile sul sito web del Comitato Etico.

Cordiali saluti.

IL PRESIDENTE
(Prof. Nicola Montanaro)

NOTA: per qualsiasi comunicazione relativa all'oggetto (compresi eventuali successivi emendamenti ed eventi avversi), è indispensabile, sia da parte dello sperimentatore che dello sponsor, fare sempre riferimento alla data della presente approvazione, nonché al numero indicato a margine dell'Oggetto.

Allegato: elenco Componenti Comitato Etico Indipendente del Policlinico S.Orsola-Malpighi

Comitato Etico Indipendente dell'Azienda Ospedaliero-Universitaria di Bologna, Policlinico S. Orsola-Malpighi

Componenti del Comitato Etico, relative qualifiche e struttura di appartenenza
Seduta del 14 Maggio 2013

Nome, cognome, qualifica	***Struttura di appartenenza***	***Presente/ Assente***
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Settore Virologia

Al Comitato Etico Indipendente
Azienda Ospedaliero-Universitaria di Bologna
Policlinico Sant'Orsola - Malpighi
Via Albertoni, 15
40138 Bologna

Oggetto: n. 41/2013/U/Tess approvato il 14/05/2013 - Sperimentazione clinica "Disordini linfoproliferativi a cellule B associati all'infezione da Epstein-Barr virus nel paziente trapiantato di cellule staminali emopoietiche" - Prot. VIRO-13

Come da Voi richiesto, Vi invio Foglio Informativo e Consenso Informato, versione 20/06/2013 modificati come richiesto.

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Lo Sperimentatore Principale

Prof.ssa Tiziana Lazzarotto

Bologna, 20 giugno 2013

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U.O. Microbiologia
Pad. 11 – S.Orsola
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E p.c. Spett.le Comitato Etico Indipendente
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Policlinico S. Orsola-Malpighi

OGGETTO: Autorizzazione alla conduzione dello studio "no profit" "Disordini linfoproliferativi a cellule B associati all'infezione da Epstein-Barr virus nel paziente trapiantato di cellule staminali emopoietiche" – Prot. VIRO-13 – Studio promosso dal Dipartimento di Medicina Specialistica, Diagnostica e Sperimentale dell'Università di Bologna/U.O. Microbiologia in collaborazione con le UU.OO. Ematologia-Cavo f.f. e Pediatria-Pession - riferimento pratica CE numero 41/2013/U/Tess

IL DIRETTORE SANITARIO

Premesso che:

- il Direttore Generale, con delibera n. 133 del 30 marzo 2012, in ragione delle funzioni connesse all'incarico, ha delegato al Direttore Sanitario il rilascio delle autorizzazioni alla conduzione degli studi clinici destinati a svolgersi all'interno delle strutture dell'Azienda Ospedaliero-Universitaria di Bologna;

Presa visione della richiesta di autorizzazione inoltrata per lo studio di cui all'oggetto;

Dato atto che lo studio:

- ha ottenuto il parere favorevole da parte del Comitato Etico Indipendente dell'Azienda nella seduta del 14 maggio 2013;

Considerato che:

- lo studio non è in contrasto né di ostacolo alle priorità assistenziali e programmatorie dell'Azienda, come dichiarato dal Direttore dell'Unità Operativa presso cui verrà condotto;

Direzione Generale
Direttore Sanitario: Dott. Mario Cavalli
Via Albertoni, 15 - 40138 Bologna
T. +39.051.636.1381 - F. +39.051.636.1202
segreteria.sanitaria@aosp.bo.it

Azienda Ospedaliero - Universitaria di Bologna
Policlinico S. Orsola-Malpighi
Via Albertoni, 15 - 40138 Bologna
T. +39.051.636.1111 - F. +39.051.636.1202
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- i benefici previsti, terapeutici ed in materia di sanità pubblica, giustificano i rischi (direttiva europea 2001/20/CE art. 3), come verificato dal Comitato Etico;

Valutati i costi correlati allo studio e le modalità di copertura economica;

Tutto ciò premesso valutato e considerato,

dichiara che **SI AUTORIZZA**

la conduzione dello studio: "Disordini linfoproliferativi a cellule B associati all'infezione da Epstein-Barr virus nel paziente trapiantato di cellule staminali emopoietiche" – Prot. VIRO-13 – Studio promosso dal Dipartimento di Medicina Specialistica, Diagnostica e Sperimentale dell'Università di Bologna/U.O. Microbiologia in collaborazione con le UU.OO. Ematologia-Cavo f.f. e Pediatria-Pession.
Direttore: Prof.ssa Maria Paola Landini
Sperimentatore principale: Prof.ssa Tiziana Lazzarotto

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Il Direttore Sanitario
Dott. Mario Cavalli

Direzione Generale
Direttore Sanitario: Dott. Mario Cavalli
Via Albertoni, 15 - 40138 Bologna
T. +39.051.636.1381 - F. +39.051.636.1202
segreteria.sanitaria@aosp.bo.it

Azienda Ospedaliero - Universitaria di Bologna
Policlinico S. Orsola-Malpighi
Via Albertoni, 15 - 40138 Bologna
T. +39.051.636.1111 - F. +39.051.636.1202
Cod. Fisc. 92038610371

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Brief communication

Successful management of EBV-PTLD in allogeneic bone marrow transplant recipient by virological–immunological monitoring of EBV infection, prompt diagnosis and early treatment

Angela Chiereghin [a], Clara Bertuzzi [b], Giulia Piccirilli [a], Liliana Gabrielli [a], Diego Squarzoni [a], Gabriele Turello [a], Martina Ferioli [c], Mariarosaria Sessa [c], Francesca Bonifazi [c], Lucia Zanoni [d], Elena Sabattini [b], Tiziana Lazzarotto [e,*]

[a] Operative Unit of Clinical Microbiology, Laboratory of Virology, St. Orsola-Malpighi University Hospital, Via Massarenti 9, 40138 Bologna, Italy
[b] Department of Specialised, Experimental, and Diagnostic Medicine, Hematopathology Section, St. Orsola-Malpighi University Hospital, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy
[c] Institute of Hematology and Medical Oncology, "L. and A. Seràgnoli", St. Orsola-Malpighi University Hospital, Via Massarenti 9, 40138 Bologna, Italy
[d] Department of Specialised, Experimental, and Diagnostic Medicine, Operative Unit of Nuclear Medicine, St. Orsola-Malpighi University Hospital, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy
[e] Department of Specialised, Experimental, and Diagnostic Medicine, Operative Unit of Clinical Microbiology, Laboratory of Virology, St. Orsola-Malpighi University Hospital, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy

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ABSTRACT

Epstein–Barr virus-related post-transplant lymphoproliferative disorder (EBV-PTLD) is an uncommon, but frequently fatal, complication after allogeneic hematopoietic stem cell transplant. Prospective post-transplant virological and immunological monitoring allowed to successfully manage a patient who developed both polymorphic and monomorphic, "diffuse large B-cell lymphoma like", as an EBV-PTLD, 65 days after allogeneic bone marrow transplant. Early detection of significant increase in EBV DNA level in patient's peripheral blood (peak of viral load equal to 119,039 copies/mL whole blood, +56 day after transplant) led to administration of pre-emptive anti-CD20 monoclonal antibody (rituximab) and close clinical monitoring. After one week, physical exam revealed laterocervical adenopathy. Histopathologic features, immunohistochemical characterization and *in situ* hybridization study allowed to establish a diagnosis of EBV-related PTLD. Immunological monitoring showed no EBV-specific T-cell responses during EBV replication, thus potentially explaining the occurrence of high EBV load with subsequent PTLD development. A total of four doses of anti-CD20 monoclonal antibody were administered and at the end of the treatment, EBV infection was cleared and imaging technique showed complete disease remission. In conclusion, the early use of anti-CD20 monoclonal antibody proved to be a safe and effective treatment strategy for EBV-PTLD. Moreover, combined virological–immunological monitoring of EBV infection may more accurately assess patients at higher risk for EBV-PTLD.

Epstein–Barr virus-related post-transplant lymphoproliferative disorder (EBV-PTLD) consists of a heterogeneous group of EBV diseases with neoplastic lymphoproliferation, developing after transplant, and caused by iatrogenic suppression of T-cell function [1]. After allogeneic hematopoietic stem cell transplant (allo-HSCT), the incidence of EBV-PTLD is lower than 2% and may increase to 10–20% in patients with established risk factors [1,2]. Prospective monitoring of EBV viremia by quantitative PCR assay is recommended for patients at high risk of PTLD, since EBV-PTLD is usually accompanied by increased EBV DNA levels in peripheral blood [1,3–5].

Here we present the successful management of a 40 year-old male who developed both polymorphic and monomorphic, "diffuse large B-cell lymphoma (DLBCL) like", EBV-PTLD, 65 days after receiving allo-HSCT for acute lymphoblastic leukemia.

In September 2013, in a routine screening, the peripheral blood showed leukocytosis with lymphocytosis, with atypical features; a

Abbreviations: EBV-PTLD, Epstein–Barr virus-related post-transplant lymphoproliferative disorder; DLBCL, diffuse large B-cell lymphoma; allo-HSCT, allogeneic hematopoietic stem cell transplant; ATG, antithymocyte globulin; GVHD, graft-versus-host-disease; EliSpot, Enzyme-linked ImmunoSPOT; ^{18}F-FDG PET/CT, ^{18}F-fluorodeoxyglucose positron emission tomography-computed tomography.

* Corresponding author.
E-mail addresses: angela.chiereghin@gmail.com (A. Chiereghin), clarabertuzzi@gmail.com (C. Bertuzzi), giulia.piccirilli@yahoo.it (G. Piccirilli), liliana.gabrielli@aosp.bo.it (L. Gabrielli), diegosquarzoni@yahoo.it (D. Squarzoni), turellogabriele@gmail.com (G. Turello), ferioli.martina@alice.it (M. Ferioli), sessamariarosaria@gmail.com (M. Sessa), francesca.bonifazi@unibo.it (F. Bonifazi), luciaironic@hotmail.it (L. Zanoni), elena.sabattini@aosp.bo.it (E. Sabattini), tiziana.lazzarotto@unibo.it (T. Lazzarotto).

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bone marrow biopsy was performed and showed the presence of an Acute pro-B Lymphoblastic Leukemia Ph+. Eight months after the initial diagnosis, the patient underwent allo-HSCT from an HLA-identical unrelated donor, at the Institute of Hematology and Medical Oncology "L. and A. Seràgnoli", St. Orsola-Malpighi University Hospital of Bologna. The source of stem cells was bone marrow. The donor was provided by the German Bone Marrow Donor Registry — DKMS Deutsche Knochenmarkspenderdatei GmbH. The donor and recipient were both positive for EBV. The conditioning regimen consisted of cyclophosphamide 60 mg/kg/day for 2 consecutive days, followed by single dose – single fraction total body irradiation, 8 Gy at a dose rate of 7 cGy/min. Low dose anti-thymocyte globulin (ATG) GRAFELON (formerly ATG-Fresenius; Neovii Biotech GmbH, Graefelfing, Germany), total 30 mg/kg, was administered over 5 days, from day −6 to −2. Total nucleated cells at the dose of 2.88×10^8/kg, CD34 positive cells at the dose of 2.05×10^6/kg and CD3 positive cells at the dose of 28.52×10^6/kg were infused. Graft-versus-host-disease (GVHD) prophylaxis consisted of Cyclosporine A and short course methotrexate (15 mg/m^2 at day +1, 10 mg/m^2 at days +3 and +6). Cyclosporine A was administered in order to maintain blood concentration ranging from 150 to 258 ng/mL. The patient did not develop acute GVHD and steroids were not administered.

Prospective virological and immunological monitoring of EBV infection was performed during the post-transplant period. Virological follow-up of EBV infection was performed every week during the first 100 days post-transplant and every 2 weeks until 180 days post-transplant. Afterwards, the patient's blood samples were processed after each examination. Specifically, routine virological monitoring was performed on whole blood samples, by a commercial quantitative real-time PCR (EBV ELITe MGB™ kit, ELITech Group, Turin, Italy), following the manufacturer's instruction. The PCR assay targets a region of the EBNA-1p gene of EBV. The analytical sensitivity of the assay is 10 copies of target DNA per amplification reaction. The lower limit of quantification (LLQ) of the assay is 225 copies/mL whole blood. PCR assay on a bioptic sample from the patient's excised lymph node was also performed. The LLQ of the assay is 10 copies/microgram DNA. Immunological monitoring was carried out by Enzyme-linked ImmunoSPOT Assay (EliSpot; EliSpot Interferon-γ Basis Kit; GenID GmbH, Strasburg, Germany) at days 48, 100, 180, 270 and 365 after transplant. The assay enumerates Interferon-gamma-secreting EBV-specific T cells (both CD4+ and CD8+ cells), at a single cell level, upon in vitro stimulation with latent and lytic viral antigens. With this method it is possible to detect one antigen specific T-cell out of 1000 cells. Two commercially available latent and lytic EBV-specific peptide mix were used (GenID GmbH, Strasburg, Germany). In particular, peptide pools derived from Epstein–Barr nuclear antigens ($EBNA_{pp}$) such as $EBNA3A_{pp}$ (amino acid (AA) 158–166, 325–333, 379–387, 458–466, 603–611) and $EBNA3C_{pp}$ (AA 258–266, 281–290), latent membrane protein 2-derived single peptide ($LMP2_p$, AA 426–434), $BRLF1_{pp}$ (AA 148–156, 134–143, 28–37), $BMLF1_{pp}$ (AA 259–267, 280–288) and $BZLF1_p$ (AA 190–197) were used. Cells were stimulated separately with latent and lytic viral antigens at 5 μg/mL or phytohemagglutinin (PHA-P) at 5 μg/mL or culture medium only (negative control). The mitogen stimulation and the negative control were included to determine general T-cell responsiveness (PHA-P ≥ 50 SFCs/200,000 lymphocytes) and background (≤10 SFCs/200,000 lymphocytes), respectively. Cellular immunity to EBV in healthy EBV-seropositive individuals was also evaluated to check each assay run. The test's procedure was conducted as described elsewhere [6]. Paraffin sections from the pathological sample were subjected to antigen retrieval and stained with monoclonal antibodies directed against the following antigens: CD20, CD10, BCL6, IRF4, and Bcl2. The *in situ* hybridization was performed using the EBV (EBER) CISH PNA Probe Y5200 (Dako), according to the manufacturer's instructions. During the immediate post-transplant period, the patient did not develop severe complications. The results obtained by virological and immunological monitoring of EBV infection and the patient's immune competence were reported in Fig. 1.

The line (·····) indicates the Lower Limit of Quantification of the real time PCR assay for the whole blood samples (225 copies/mL). The line (·····) indicates the EliSpot assay cut-off for positive response to EBV (≥5 SFCs/200,000 lymphocytes, calculated using the formula "sample — negative control"). The lines with filled and open circles represent virological and immunological monitoring, respectively. In the upper area of the graph, the total lymphocyte count (10^3/mmc, reference interval 0.80–4.32) obtained at each virological–immunological time point was reported.

Thirty-three days post-transplant, the patient resulted positive for EBV-DNA (375 copies/mL) on routine virological monitoring. At +40 day after allo-HSTC, EBV DNAemia increased (11,150 copies/mL) and at the next visit, EBV-specific T-cell responses by EliSpot assay were also assessed. In particular, at +48 day after allo-HSTC, the presence of general T-cell responsiveness (response to mitogen stimulation, PHA-P ≥ 50 SFCs/200,000 lymphocytes) and lack of specific T-cell responses against both EBV latent and lytic antigens (≤5 SFCs/200,000 lymphocytes, "sample — negative control") were detected by EliSpot assay; EBV DNAemia was of 28,002 copies/mL. The EBV DNAemia continued to increase (88,695 copies/mL, +54 day after allo-HSTC) reaching the peak at +56 day after transplant (119,039 copies/mL). The patient started with anti-CD20 monoclonal antibody (rituximab) consisting in 4 administrations, at a dose of 375 mg/m^2/week, on day

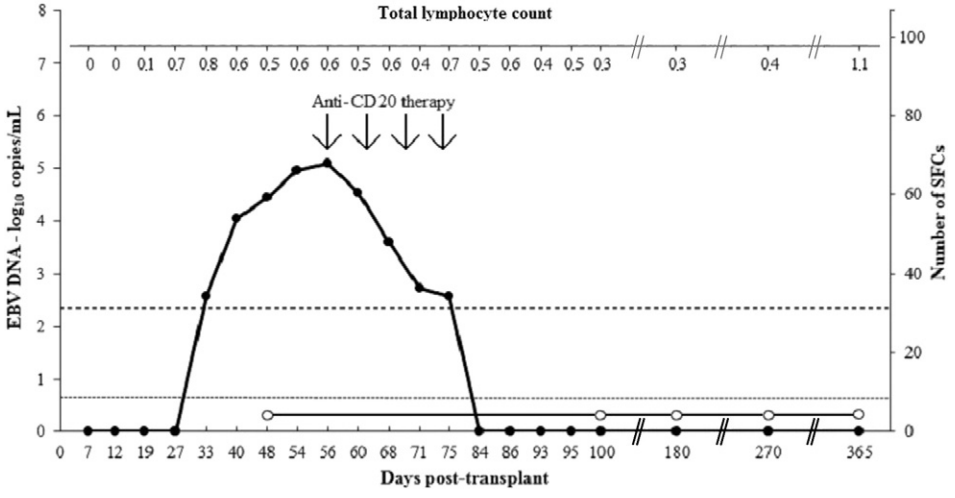

Fig. 1. Results of the virological–immunological monitoring of EBV infection and total lymphocyte count during the first year post-transplant.

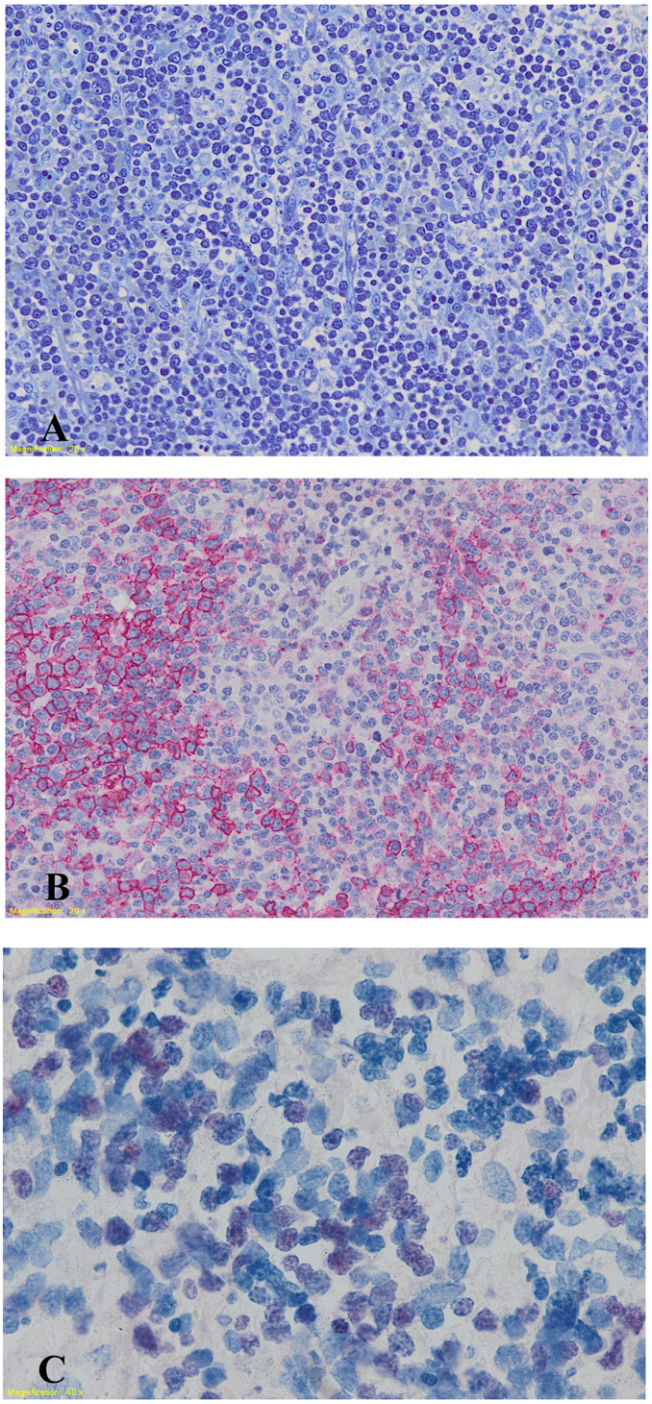

Fig. 2. Histology and immunohistochemistry of the lymph node. A. Polymorphic cell population with diffuse growth pattern, composed by lymphoid cells and plasma cells (Giemsa). B. Partial immunoreactivity for CD20, probably due to the previous therapy with anti-CD20 monoclonal antibody (rituximab). C. *In situ* hybridization shows EBV-RNA integration in the pathologic cell population.

+56. After one week, the physical examination revealed a cervical lymph node enlargement which led to lymph node excision at day +65. The lymph node structure was affected by an atypical polymorphic cell population with diffuse growth pattern, composed by lymphoid cells of variable size, with a moderate amount of large elements, with round nuclei and multiple peripheral nucleoli, as well as plasma cells (Fig. 2A). At immunohistochemistry the large cells mainly showed a non-germinal centre B cell phenotype (CD20+, CD10−, BCL6−) with expression of Bcl2 and IRF4 and some MYC protein positive elements, while the plasma cells/plasmacytoid elements were CD20−/IRF4+ (Fig. 2B). *In situ* hybridization assay showed EBV-RNA integration in the pathologic cell population (Fig. 2C).

Upon these immuno-morphologic features, a diagnosis of an EBV-related PTLD was rendered with polymorphic and monomorphic DLBCL-like coexisting areas. In the meanwhile, PCR assay of a bioptic sample of the same lymph node revealed a high level of EBV-DNA (1,113,186 copies/microgram DNA), this result confirmed the EBV pathogenesis of PTLD. The patient was subjected to whole body ^{18}F-fluorodeoxyglucose (FDG)-positron emission tomography-computed tomography (PET/CT), which showed positive uptake in multiple lymph nodes in right and left laterocervical region together with presacral region (Fig. 3A). During the treatment, PCR assay revealed a decrease in EBV-DNA and at the end of the therapy the patient achieved EBV DNA negativity. Two months after the EBV-PTLD diagnosis and four doses of anti-CD20, ^{18}F-FDG PET/CT showed a complete disease remission (Fig. 3B).

EliSpot assay showed response to mitogen stimulation and nonresponse to EBV stimulation at all other post-transplant specific timepoints in which it was performed (+100, +180, +270 and +365 days after transplant) (Fig. 1). Serum immunoglobulin (Ig) levels – IgG, IgA and IgM – were determined routinely during the post-transplant period (data not shown). In occasion of the last follow-up visit on +365 day after allo-HSCT, the three serum immunoglobulin concentrations were lower than the reference intervals. Specifically, serum IgG, IgA and IgM levels were equal to 356 ng/mL (reference interval 700–1600), 48 ng/mL (reference interval, 70–400) and 25 ng/mL (reference interval, 40–230), respectively.

Here, we have presented a case of successful treatment of EBV-PTLD with anti-CD20 monoclonal antibody after allogeneic bone marrow transplant. The patient developed an early EBV-related PTLD (*i.e.* two months after allo-HSCT; <1 year post-transplant). This is in accordance with the published literature, in fact particularly high rates of PTLD during the first 6 months after transplant are observed and the EBV genome is found in more than 90% of B-cell PTLD occurring during the first year after transplantation [2,7]. Moreover, the patient had two known risk factors for PTLD, specifically he had received a graft from an unrelated donor and antithymocyte globulin during the conditioning regimen. However the antithymocyte globulin, used at a low dose, has shown no increase of viral infections – EBV-related PTLD included – in over a decade of use at our Institution [8]. Prospective virological monitoring of EBV infection allowed early detection of significant increase in EBV DNA level in the patient's peripheral blood. Patient was closely monitored for symptoms attributable to EBV disease and pre-emptive anti-CD20 monoclonal antibody was administered at the peak of viral load. Nevertheless, after one week, the progression of EBV infection to overt disease was observed. A total of four doses of anti-CD20 monoclonal antibody were administered. Treatment was well tolerated and no significant adverse events attributed to the drug occurred. A decrease in EBV-DNA load of 2 log of magnitude in the first two weeks of treatment was observed. Furthermore, EBV infection was cleared at the end of the therapy. The early effects of the treatment were already evident in immunohistochemistry, in fact we found only a partial CD20 immunoreactivity due to the selective effect of anti-CD20 as monoclonal antibody. A complete disease remission, two months after the EBV-PTLD diagnosis and four doses of anti-CD20 monoclonal antibody was observed and whole blood samples tested negative for EBV DNA. The occurrence of high EBV load with subsequent PTLD development could be explained by the lack of EBV-specific immune responses observed in the patient during EBV replication. In fact, several studies have reported that the risk for EBV-PTLD is correlated with lack or delay of EBV-specific immune reconstitution after stem cell transplant [9–11]. Furthermore, immunological monitoring showed no EBV-specific cellular immune reconstitution during the remaining post-transplant period. Moreover, a moderate immunoglobulin deficiency – IgG, IgA and IgM – was also observed at one year post-transplant. This result is in accordance with other findings, in particular Bordon et al. [12] observed that in pediatric allo-HSCT recipients, an adequate immune reconstitution after the use of

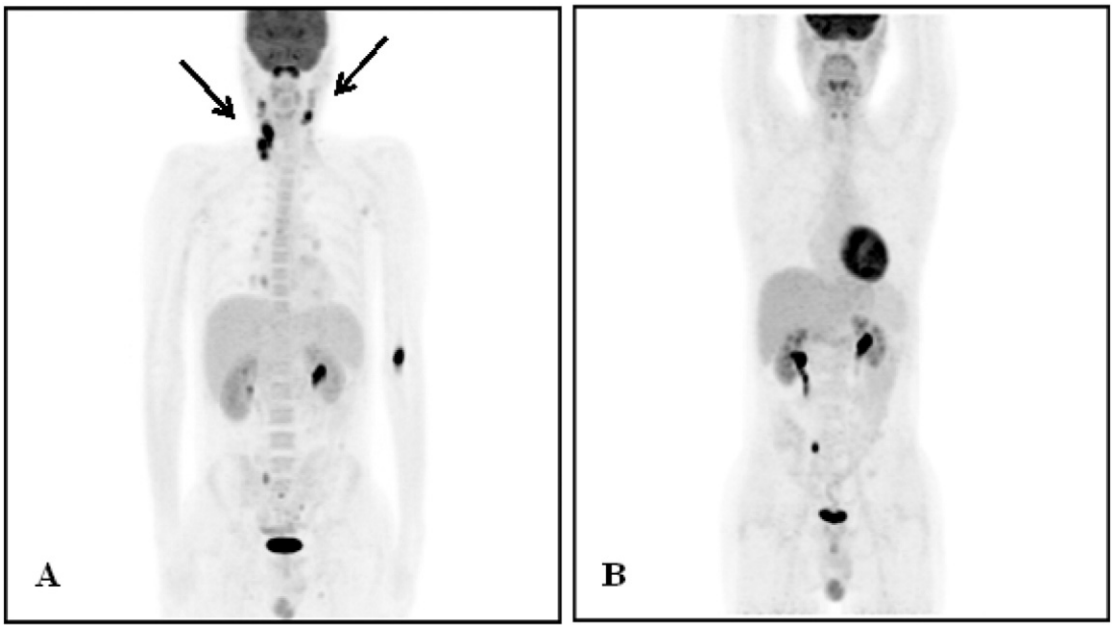

Fig. 3. Baseline (at diagnosis) ^{18}F-FDG PET/CT (A) and post-treatment ^{18}F-FDG PET/CT (B). **A:** ^{18}F-FDG PET/CT shows pathological uptake in laterocervical lymph nodes both right (stations II–III–IV; SUVmax value 12.9) and left side (stations I–II; SUVmax value 13.5) and in presacral node (SUVmax value 7.4). **B:** Post-treatment ^{18}F-FDG PET/CT shows a complete metabolic response (no pathological uptake).

anti-CD20 monoclonal antibody (rituximab) early after allo-HSCT can take years. No other episode of EBV reactivation was observed and EBV-PTLD persists in remission 10 months following the initial diagnosis. At 9 months post-transplant, the patient developed limited chronic GVHD that did not require immunosuppression therapy. To date, the patient is alive and well and regularly seen for follow-up.

In conclusion, the early use of the anti-CD20 monoclonal antibody (rituximab) has proven to be a safe and effective treatment strategy for EBV-PTLD, confirming the literature data [13–15]. Furthermore, our data showed the potential usefulness of measuring EBV blood replication combined with the specific viral cellular-immunity in allo-HSCT recipients in order to identify the patients at higher risk for EBV-PTLD who would benefit from more appropriate pre-emptive interventions.

Currently, a prospective clinical trial is in progress at our University Hospital in order to assess the real value of the aforementioned monitoring strategy for EBV infection in both adult and pediatric allo-HSCT transplant recipients.

Conflict of interest

The authors declare no conflict of interest.

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Prospective Epstein–Barr virus-related post-transplant lymphoproliferative disorder prevention program in pediatric allogeneic hematopoietic stem cell transplant: virological monitoring and first-line treatment

A. Chiereghin, A. Prete, T. Belotti, D. Gibertoni, G. Piccirilli, L. Gabrielli, A. Pession, T. Lazzarotto. Prospective Epstein–Barr virus-related post-transplant lymphoproliferative disorder prevention program in pediatric allogeneic hematopoietic stem cell transplant: virological monitoring and first-line treatment.
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Abstract: *Background.* In 28 pediatric allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients, we aimed to evaluate: (i) the impact of routine Epstein–Barr virus (EBV) DNA monitoring on the development of EBV-related post-transplant lymphoproliferative disorder (EBV-PTLD); (ii) the incidence of EBV infection and the potential risk factors; and (iii) the suitability of whole blood (WB) as clinical specimen to monitor the risk of patients to develop EBV-PTLD.
Methods. Quantitative real-time polymerase chain reaction assay was performed on WB samples for all patients. EBV DNA quantification also in peripheral blood mononuclear cells (PBMCs) samples was adopted for the patients at higher risk of developing EBV-PTLD (≥10,000 copies/mL WB).
Results. High EBV DNAemia levels were observed in 37.5% of the actively infected recipients (57.1%). Severe aplastic anemia, matched-unrelated donor transplant, the reduced-intensity conditioning regimen and, to a lesser extent, the *in vivo* T-cell depletion with anti-thymocyte immunoglobulin were associated with high viral load. A significant correlation between EBV DNA levels in WB and PBMC samples was obtained ($r = 0.755$, $P < 0.001$). A similar kinetics of EBV DNA in the 2 blood compartments was observed. Clinically, both specimen types appeared to be equally informative to assess the risk of patients to develop PTLD. On the basis of EBV DNAemia levels, in 3 patients (10.7%) immunosuppressive therapy was reduced and 1 patient (3.5%) received early treatment for probable EBV disease. No patients developed EBV-PTLD.
Conclusion. WB proved to be a suitable clinical specimen to monitor EBV DNA load after allo-HSCT for the management of EBV infection and PTLD prevention.

A. Chiereghin[1], A. Prete[2], T. Belotti[2], D. Gibertoni[3], G. Piccirilli[1], L. Gabrielli[1], A. Pession[2], T. Lazzarotto[4]

[1]Operative Unit of Clinical Microbiology, Laboratory of Virology, St. Orsola-Malpighi University Hospital, Bologna, Italy, [2]Pediatric Oncology and Haematology Unit "Lalla Seràgnoli", Department of Pediatrics, St. Orsola-Malpighi University Hospital, Bologna, Italy, [3]Department of Biomedical and Neuromotor Sciences, Unit of Hygiene and Biostatistics, University of Bologna, Bologna, Italy, [4]Department of Specialised, Experimental, and Diagnostic Medicine, Operative Unit of Clinical Microbiology, Laboratory of Virology, St. Orsola-Malpighi University Hospital, University of Bologna, Bologna, Italy

Key words: pediatric allogeneic hematopoietic stem cell transplant; EBV-related post-transplant lymphoproliferative disorder; preemptive therapy; first-line treatment of PTLD; risk factors; kinetics of EBV DNA load

Correspondence to:
Tiziana Lazzarotto, PhD, Laboratory of Virology, St. Orsola-Malpighi University Hospital, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy
Tel: +39 051.2143360
Fax: +39 051.307397
E-mail: tiziana.lazzarotto@unibo.it

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Epstein–Barr virus (EBV) is a ubiquitous γ-herpesvirus with tropism for B lymphocytes that infects at least 90% of the population worldwide, in which it establishes a lifelong asymptomatic infection (1). In recipients of allogeneic hematopoietic stem cell transplant (allo-HSCT), EBV infection may progress to onset of

a post-transplant lymphoproliferative disorder (PTLD), a complication that is the result of the outgrowth of EBV-infected B cells that, in immunocompetent individuals, is normally controlled by an effective EBV-specific cytotoxic T-cell response (2, 3). PTLD typically occurs within the first 6 months after transplantation and, in >90% of cases, PTLD is EBV positive (4, 5). Risk factors for development of this complication include type of donor, T-cell depletion, use of anti-thymocyte immunoglobulin (ATG), EBV serology mismatch between donor and recipient, and splenectomy (6). In a recent large, multicenter and retrospective analysis, the overall EBV-related PTLD frequency observed was of 3.22%, ranging from 1.16% for matched-family donor recipients to 11.24% in mismatched-unrelated donor recipients (7). Although several studies have convincingly demonstrated that the development of EBV-PTLD, both after solid organ and stem cell transplant (SCT), is mostly accompanied by a significant increase of EBV DNA levels in the blood of patients (8–13), to date, the choice of optimal clinical specimen – whole blood (WB) or peripheral blood mononuclear cells (PBMCs) – for the monitoring of EBV infection in transplanted patients is a matter of debate (14–17). In addition, no international consensus exists on what EBV DNA threshold level is predictive for developing PTLD and then for the need of preemptive therapy. In fact, various data about this issue have been reported and have been related to local experiences (6, 18–23). First-line treatment of PTLD includes: (i) anti-CD20 monoclonal antibody rituximab 375 mg/m^2, weekly doses – the number of doses is assessed locally on the basis of changes in EBV DNA load; (ii) reduction of immunosuppressive therapy, if possible; (iii) donor lymphocytes infusion; and (iv) donor EBV-cytotoxic T-lymphocytes infusion, if available (6).

The aims of the study were to evaluate: (i) the impact of routine EBV DNA monitoring and first-line treatment of PTLD on the development of EBV-related disease in pediatric allo-HSCT recipients; (ii) the incidence of EBV infection and the potential risk factors; and (iii) the kinetics of EBV DNA in 2 different blood compartments – WB and PBMCs – in order to establish if WB is a suitable clinical specimen to monitor the risk of developing EBV-PTLD in allo-HSCT recipients.

Materials and methods

Study population and transplant characteristics

The study population included 28 consecutive pediatric patients who underwent allo-HSCT at the Operative Unit of Pediatrics, Oncology, Haematology, and Stem Cell Transplantation Program, of the St. Orsola-Malpighi University Hospital, Bologna, Italy, between March 2012 and November 2013. The population was made up of 20 male and 8 female pediatric patients, with a mean age of 9.4 years (range 9 months–18.4 years). The study population's transplant characteristics are reported in Table 1. Most patients (42.8%) underwent transplant for acute lymphoblastic leukemia. The donor type was a matched-unrelated donor (MUD) for 18 allo-HSCT (64.3%) and a related-donor type for 10 (35.7%) patients; in all transplants the donor was human leukocyte antibody (HLA)-identical sibling. The source of stem cells was bone marrow in 85.8% of transplants. At the time of transplantation, the EBV serostatus of donor/recipient (D/R) was as follows: D+/R+ in 12/28 patients (42.8%), D+/R− in 1/28 patients (3.6%), and D−/R+ in 2/28 patients (7.1%). The serology of the donor was not available in 13 cases (46.5%); the respective recipients were: 10 EBV seropositive and 3 EBV seronegative. The conditioning regimen was reduced-intensity conditioning (RIC) for 11 patients and myeloablative for 17 patients. A total of 20 patients (71.4%) underwent *in vivo* T-cell depletion to reduce rejection and graft-versus-host disease (GvHD): all 18 MUD transplants and 2 of 10 HLA-identical sibling transplants. In 17 of 20 patients (Patients number 1, 8, 11, 14–25, 27, 28; Table 1) rabbit ATG (Thymoglobulin®, Pasteur-Mérieux Connaught, Lyon, France) in a dose ranging from 2–3.75 mg/kg was used for a different period of time (range 3–5 days). Three patients received rabbit ATG (ATG-Fresenius®, Fresenius Ag, Gräfelfing, Germany) for 3 consecutive days at the dose of 5 mg/kg (Patients number 4, 7, 26; Table 1). In most cases, GvHD prophylaxis consisted of an association of cyclosporine and methotrexate (n = 18). During the engraftment, analysis of chimerism was performed by short tandem repeats–polymerase chain reaction (PCR) method on peripheral blood samples. Full donor chimerism in peripheral blood was detected in patients at a median time of 20 days (range 12–35). Furthermore, from day +30, chimerism status was also analyzed on bone marrow samples that were collected every 3–6 months during the first 2 years post transplant. All the post-transplant days when full donor chimerism was detected in patients' peripheral blood and bone marrow samples are reported in Table 1.

Study design

Virological monitoring of EBV infection was performed on WB samples using a commercial quantitative real-time PCR. Follow-up of EBV infection was

Characteristics of 28 patients and results of virological monitoring of Epstein–Barr virus (EBV) infection

Pt	Age, years	Gender	Primary disease	Donor type	Graft origin	EBV serology	Conditioning regimen	T-cell depletion	GvHD prophylaxis	EBV DNAemia copies/mL whole blood	Chimerism analysis day after-transplant	
											Peripheral blood	Bone marrow
16	2	M	DBA	MUD	BM	D+/R−	RIC regimen	*in vivo*	CyA + MTX	min-max 278–896,096	CC + 17	CC + 50
17	11.2	F	SAA	MUD	BM	D−/R+	RIC regimen	*in vivo*	CyA + MTX	min- max <225–127,410	CC + 12	CC + 55
18	12.8	F	SAA	MUD	BM	D−/R+	RIC regimen	*in vivo*	CyA + MTX	min-max <225–127,869	CC + 21	CC + 60
19	4.9	M	ALL	MUD	BM	D?/R−	Busulfan-based MAC regimen	*in vivo*	CyA + MTX	min-max <225–512,905	CC + 22	CC + 40
22	6.8	M	FA	MUD	BM	D?/R+	RIC regimen	*in vivo*	CyA + MTX	min-max <225–10,477	Graft rejection	-
25	16.3	F	SAA	MUD	BM	D?/R+	RIC regimen	*in vivo*	CyA + MTX	min-max 6723–81,894	CC + 19	CC + 33
20	3.2	M	LCH	MUD	BM	D?/R−	Busulfan-based MAC regimen	*in vivo*	CyA + MTX	min-max <225–3,630	CC + 26	CC + 42
1	6.5	M	TM	MUD	BM	D+/R+	RIC regimen	*in vivo*	CyA + MTX	min-max <225–2918	CC + 25	CC + 42
2	10	M	ALL	RS	BM	D+/R+	Busulfan-based MAC regimen	No	Only CyA	min-max <225–494	CC + 18	CC + 35
4	11.4	F	AML	MUD	PBSC	D?/R+	Busulfan-based MAC regimen	*in vivo*	CyA + MTX	min-max <225–817	CC + 21	CC + 32
5	17.5	F	ALL	RS	BM	D+/R+	Busulfan-based MAC regimen	No	CyA + MTX	min-max <225–1013	CC + 25	CC + 42
7	7.3	M	ALL	MUD	PBSC	D+/R+	TBI-based MAC regimen	*in vivo*	CyA + MTX	min-max <225–3322	CC + 19	CC + 42
12	10	M	ALL	RS	BM	D+/R+	Busulfan-based MAC regimen	No	Only CyA	min-max <225–2349	CC + 15	CC + 31
14	7.7	M	ALL	MUD	BM	D?/R+	Busulfan-based MAC regimen	*in vivo*	CyA + MTX	min-max <225–4237	CC + 14	CC + 33
15	18.4	F	SAA	RS	BM	D+/R+	RIC regimen	*in vivo*	CyA + MTX	min-max <225–1608	CC + 20	CC + 32
26	8.9	M	SCD	RS	BM	D+/R+	RIC regimen	*in vivo*	Only CyA	min-max <225–471	CC + 21	CC + 50
3	8.1	M	ALL	RS	BM	D+/R+	TBI-based MAC regimen	No	Only CyA	Negative	CC + 15	CC + 30

Table 1 Continued

Pt	Age, years	Gender	Primary disease	Donor type	Graft origin	EBV serology	Conditioning regimen	T-cell depletion	GvHD prophylaxis	EBV DNAemia copies/mL whole blood	Chimerism analysis day after-transplant	
											Peripheral blood	Bone marrow
13	13.4	M	ALL	RS	BM	D+/R+	Busulfan-based MAC regimen	No	Only CyA	Negative	CC + 19	CC + 37
21	6.2	M	AML	MUD	CB	D?/R+	Busulfan-based MAC regimen	*in vivo*	CyA + Cortisone	Negative	CC + 21	CC + 42
27	13.6	F	ALL	MUD	BM	D?/R+	Busulfan-based MAC regimen	*in vivo*	CyA + MTX	Negative	CC + 18	CC + 34
9	17	M	ALL	RS	BM	D+/R+	Busulfan-based MAC regimen	No	Only CyA	Negative	CC + 15	CC + 30
10	6.7	M	ALL	RS	BM	D+/R+	Busulfan-based MAC regimen	No	Only CyA	Negative	CC + 15	CC + 48
23	3.8	M	DBA	MUD	BM	D?/R+	RIC regimen	*in vivo*	CyA + MTX	Negative	CC + 25	Death before analysis
24	1	M	FHL	MUD	BM	D?/R−	RIC regimen	*in vivo*	CyA + MTX	Negative	CC + 35	MC + 271
28	0.8	M	AML	MUD	CB	D?/R+	Busulfan-based MAC regimen	*in vivo*	CyA + MMF + Cortisone	Negative	CC + 35	CC + 62
11	9.3	M	ALL	MUD	BM	D+/R+	Busulfan-based MAC regimen	*in vivo*	CyA + MTX	Negative	CC + 19	CC + 40
6	10.8	M	TM	RS	BM	D?/R+	RIC regimen	No	Only CyA	Negative	CC + 25	CC + 82
8	10	F	AML	MUD	BM	D?/R+	Busulfan-based MAC regimen	*in vivo*	CyA + MTX	Negative	CC + 32	CC + 82

Patients were divided into 3 groups on the basis of the viral load detected during the post-transplant follow-up of EBV infection: high viral load (≥10,000 copies/mL whole blood [WB]), low viral load (<10,000 copies/mL WB), and negative results for EBV DNAemia.
Pt, patient; GvHD, graft-versus-host disease; M, male; DBA, Diamond Blackfan anemia; MUD, matched-unrelated donor; BM, bone marrow; D, donor; R, recipient; +, positive; −, negative; RIC, reduced-intensity conditioning; CyA, cyclosporine; MTX, methotrexate; min, minimum; max, maximum; CC, complete chimerism; F, female; SAA, severe aplastic anemia; ALL, acute lymphoblastic leukemia; D?, donor serology not available; MAC, myeloablative conditioning; FA, Fanconi anemia; LCH, Langerhan cell histiocytosis; TM, thalassemia major; RS, related sibling; AML, acute myeloid leukemia; PBSC, peripheral blood stem cells; TBI, total body irradiation; SCD, sickle cell disease; CB, cord blood; FHL, familial hemophagocytic lymphohistiocytosis; MC, mixed chimerism; MMF, mycophenolate mofetil.

Table 1

performed every week during the first 100 days post transplant and every 2 weeks until 180 days post transplant. Afterward, blood samples were processed if clinically indicated. In the case of EBV DNAemia, monitoring was performed weekly for at least 1 month. Based on previous studies (24, 25) and on Italian Group of Bone Marrow Transplantation (GITMO) guidelines for the diagnosis and treatment of EBV-PTLD in HSCT recipients, any values of EBV DNAemia ≥10,000 copies/mL were defined as high viral load. For the patients at higher risk of developing EBV-PTLD, that is, patients who showed high viral load, EBV DNA load in PBMC samples was also prospectively monitored starting from the next visit. In addition, all patients were monitored for cytomegalovirus (CMV) infection during the post-transplantation period. CMV real-time PCR was performed weekly during the first 3 months and then monthly until the 6th month. Afterward, blood samples were processed if clinically indicated.

The management of EBV infection for patients with any value of EBV DNAemia ≥10,000 copies/mL was defined at individual patient level. The first-line management of EBV infection consisted of reduction in the immunosuppressive therapy. Anti-CD20 monoclonal antibody rituximab (4 administrations, at dose of 375 mg/m^2/week) was administered when reducing immunosuppression alone was not sufficient to control EBV DNAemia or when symptoms suggested EBV-related disease. Definitions of probable and proven EBV disease that were adopted in the study are those reported in the European Conference on Infections in Leukemia (ECIL) guidelines (6).

Real-time PCR for EBV DNA quantification

Real-time PCR for EBV DNA quantification was performed (EBV ELITe MGB™ kit, ELITech Group, Turin, Italy). Blood samples were collected in ethylenediamine-tetraacetic acid-anticoagulated tubes. DNA was extracted from 200 μL of WB using the QIASymphony^{sp} instrument (Qiagen GmbH, Hilden, Germany), eluted in 90 μL of elution buffer, and 20 μL were processed for EBV DNA quantification. DNA was extracted from 2×10^5 PBMCs using the NucliSens easyMAG System (bioMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions; half of the eluated volume corresponding to 1×10^5 PBMCs was used as input DNA in each real-time PCR. Amplification, detection, and analysis were performed using the ABI PRISM 7300 platform (Life Technologies, Foster City, California, USA). The PCR assay targets a region of the EBNA-1 gene of EBV. The analytical sensitivity of the assay is 10 copies of target DNA per amplification reaction. The lower limit of quantification (LLQ) of the assay is 225 copies/mL WB and 11 copies/1×10^5 PBMCs. EBV DNA load was reported as quantities of copies/mL and copies/1×10^5 PBMCs.

Statistical analysis

Comparisons between patient-related and transplant-related characteristics and EBV DNAemia were evaluated using chi-square test of independence. Patients with low EBV DNA levels (<10,000 copies/mL WB) or without active EBV infection were compared with patients who showed infection with high EBV DNA load (≥10,000 copies/mL WB). *P*-values <0.05 were considered statistically significant. The correlation between $\log_{10}$ copies of EBV DNA in WB and PBMC samples was obtained using weighted Pearson correlation to take into account the different number of subjects' repeated measures.

Results

The 28 pediatric allo-HSCT recipients enrolled were monitored for EBV infection for a median time of 7.1 months post transplant (range 1–22 months). A total of 353 WB and 88 PBMC samples were processed; the median number of paired WB and PBMC samples analyzed per patient was 15 (range 11–19).

Sixteen of 28 patients (57.1%) developed a post-transplant EBV infection: 11 patients who underwent allo-HSCT from MUDs (61.1%, 11/18 patients) and 5 patients transplanted from related donors (50%, 5/10 patients). Three of 16 patients (18.7%) developed primary EBV infection, and 13/16 (81.3%) patients developed EBV reactivation/reinfection. Therefore, in our study population, the overall incidence of post-transplant primary EBV infection and EBV reactivation/reinfection was equal to 10.7% and 46.4%, respectively.

For 12 of 28 patients (42.9%), WB samples tested negative for EBV DNA over the entire follow-up. Among the 16 patients with an active post-transplant EBV infection, 10 patients (62.5%) showed low values of EBV DNAemia (<10,000 copies/mL WB), whereas 6 patients (37.5%) showed values of EBV DNAemia ≥10,000 copies/mL WB. The median peak of viral load in patients with low and high values of EBV DNAemia was 1978 copies/mL (range: 471–4237 copies/mL) and 127,639 copies/mL (range: 10,477–896,096 copies/mL), respectively. The median time at which EBV DNAemia

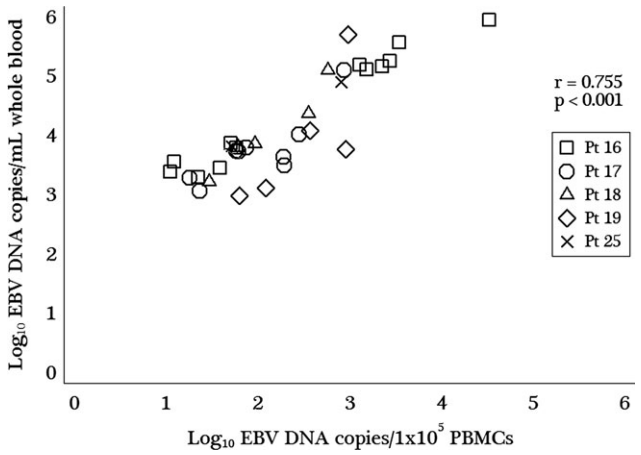

Fig. 1. Correlation between Epstein–Barr virus (EBV) DNA levels in 33 whole blood and periphal blood stem cells (PBMCs) samples of pediatric allogeneic hematopoietic stem cell transplant recipients. Pt, patient.

was first detected was 47 days (range 13–354 days) post transplant. The results obtained by virological monitoring, along with the range of EBV DNAemia values detected per patient, are reported in Table 1. In total, 88 paired WB and PBMC samples, collected from the 6 patients with high EBV DNAemia levels, were processed. Twenty-seven of 88 samples (30.7%) were negative and 58 samples (65.9%) were positive for EBV DNA both in WB and PBMC samples; 3 (3.4%) samples were discordant. Among the 58 positive samples: 33 samples (56.9%) were positive with a quantitative result; 12 samples (20.7%) tested positive but below the LLQ of the PCR assay, and 13 samples (22.4%) were positive with a quantitative result in WB and positive, but below the LLQ of the PCR assay, in PBMC samples. The 33 of 88 samples that resulted positive with a quantitative result in both the 2 blood compartments were taken into consideration for the correlation analysis. The correlation coefficient obtained was $r = 0.755$ ($P < 0.001$) (Fig. 1). EBV DNA kinetics in WB and PBMC samples were analyzed in all 6 patients. Representative EBV DNA kinetics in the 2 blood compartments of 4 of 6 patients are reported in Figure 2.

Chi-square test of independence showed a significant association between EBV DNAemia levels ≥10,000 copies/mL and both transplants from unrelated donors and RIC regimen ($P = 0.039$ and $P = 0.013$, respectively) (Table 2). Moreover, an association between viral load and underlying disease was found ($P = 0.027$), with patients suffering from severe aplastic anemia (SAA) showing a significantly higher incidence of EBV infection with high viral load. A milder association between the development of EBV infection with high viral load and *in vivo* T-cell depletion with ATG was observed ($P = 0.081$). No relationship was observed between the origin of the graft; the development of post-transplant CMV infection; the acute (<100 days post transplant) and chronic GvHD (≥100 days post transplant; data were available for only 25 patients as 3 of 28 patients died after 100 days post transplant); the EBV serostatus of the recipients; and the development of EBV infection with high EBV DNAemia levels (Table 2). None of the patients underwent splenectomy; therefore, comparison between this clinical parameter and EBV DNAemia could not be evaluated.

Regarding the management of EBV DNAemia and PTLD prevention, among the 10 patients who maintained viral loads <10,000 copies/mL WB, in 9/10 patients (90%) EBV clearance occurred spontaneously and in 1/10 patients (10%) immunosuppression was reduced at the peak of viral load (D?/R−); the patient achieved EBV DNA negativity in both the 2 blood compartments 20 days after reduction of the immunosuppressive therapy. Among the 6 patients with EBV DNA load ≥10,000 copies/mL WB, 5 (83.3%) had no symptoms nor signs of EBV-PTLD, and 1 had a probable EBV disease (16.7%), defined as lymphadenopathy with high EBV blood load, in the absence of other etiologic factors or established diseases (6). In particular, among the 5 asymptomatic patients, 2 patients (40%) controlled EBV DNAemia spontaneously, and in 3 patients (60%) the immunosuppressive therapy was reduced in the presence of median EBV DNA levels in WB of 512,905 copies/mL and of 1100 copies/1 × 10^5 PBMCs. A decrease in EBV DNA levels in both blood compartments was observed in all 3 patients who achieved low EBV DNA levels at a median time of 9 days after the reduction in the immunosuppressive therapy. Anti-CD20 monoclonal antibody rituximab (4 administrations, at dose of 375 mg/m^2/week) was administrated at day +115 after HSCT to the patient with probable EBV disease. In the first week of treatment, a decrease in EBV DNA load at 1-log of magnitude was observed with subsequent symptom regression at the end of the therapy. The reduction in immune suppression and the administration of anti-CD20 monoclonal antibody rituximab were carried out at the peak of DNA levels that were reached simultaneously in both the 2 blood compartments.

Among the 28 patients in the study, 8 patients (28.6%) died at a median time of 7 months (range 1–11) after allo-HSCT. The causes of death were relapse of the original disease ($n = 4$), cerebral hemorrhage ($n = 2$), and pulmonary infection ($n = 2$). The remaining 20 patients (71.4%) were alive and well.

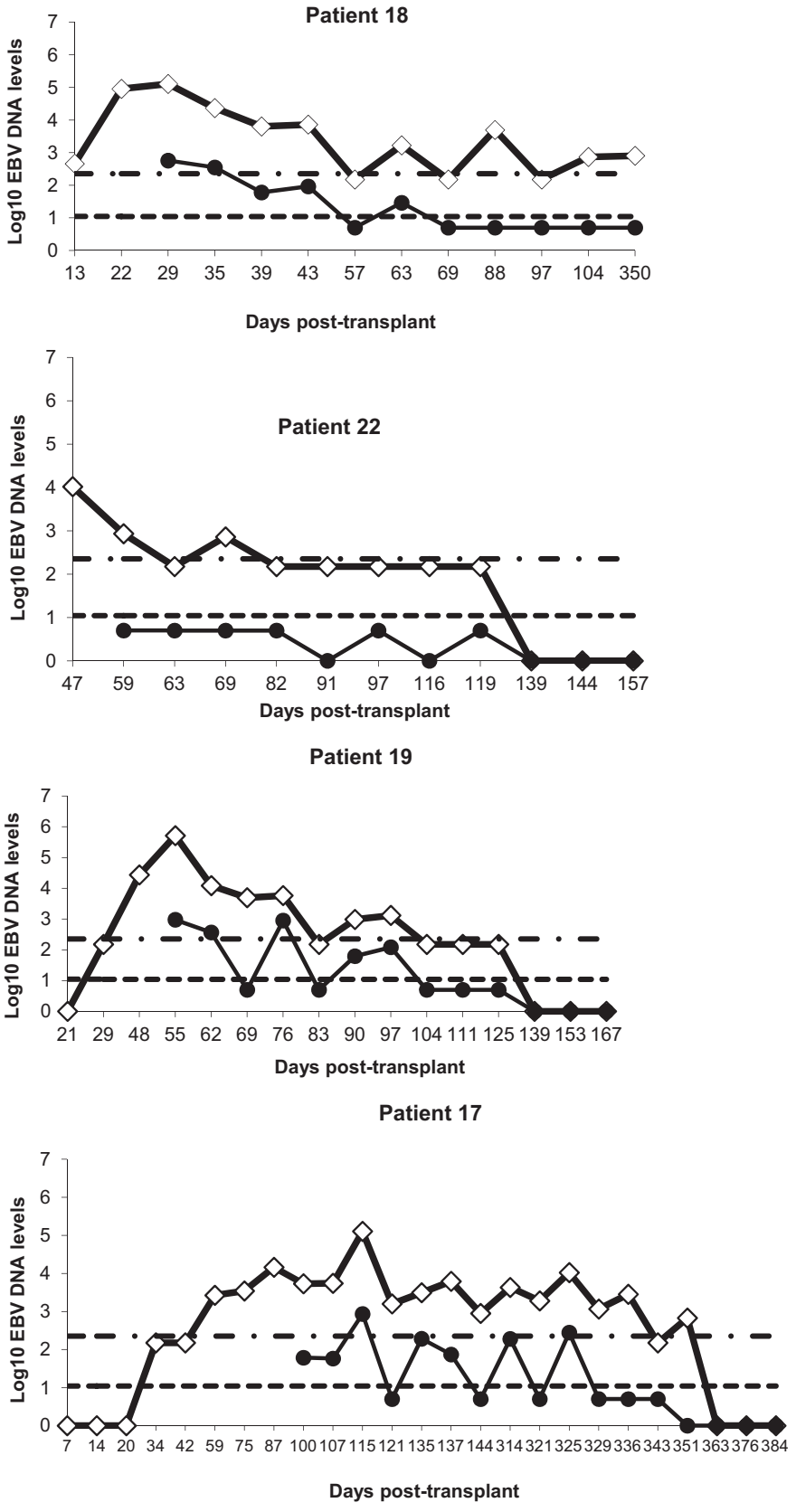

Fig. 2. Representative Epstein–Barr virus (EBV) DNA kinetics in whole blood (WB) and peripheral blood mononuclear cells (PBMC) samples of 4 allogeneic hemopoietic stem cell transplant recipients (Patients 18, 22, 19, and 17) with high EBV DNAemia levels in the post-transplant period. The line (-·-·-) indicates the lower limit of quantification (LLQ) of the real-time polymerase chain reaction (PCR) assay for WB samples. The line (----) indicates the LLQ of the real-time PCR assay for PBMC samples. Log_{10} EBV DNA levels in WB and in PBMC samples were represented with open squares and filled circles, respectively.

Patient characteristics and risk factors for Epstein–Barr virus (EBV) infection

	Total	EBV DNAemia Negative or <10,000 copies/mL Whole blood	EBV DNAemia ≥10,000 copies/mL Whole blood	*P*-value
Number of patients (%)	28 (100)	22 (78.6)	6 (21.4)	
Gender: male/female	20/8	17/5	3/3	0.190
Primary disease				
ALL	12 (41.4)	11 (91.7)	1 (8.3)	**0.027**
SAA	4 (13.8)	1 (25.0)	3 (75.0)	
AML	4 (17.2)	4 (100)	0	
Other[1]	8 (27.6)	6 (75.0)	2 (25.0)	
Donor type				
Unrelated (MUD)	18 (64.3)	12 (66.7)	6 (33.3)	**0.039**
Related (Sibling)	10 (35.7)	10 (100)	0	
Graft origin				
Bone marrow	24 (85.7)	18 (75.0)	6 (25.0)	0.529
Peripheral blood	2 (7.1)	2 (100)	0	
Cord blood	2 (7.1)	2 (100)	0	
Conditioning regimen				
Myeloablative[2]	17 (60.7)	16 (94.1)	1 (5.9)	**0.013**
Reduced-intensity	11 (39.3)	6 (54.5)	5 (45.5)	
In vivo T-cell depletion with ATG				
Yes	20 (71.4)	14 (70.0)	6 (30.0)	0.081
No	8 (28.6)	8 (100)	0	
CMV infection				
Yes	12 (42.8)	9 (75.0)	3 (25.0)	0.690
No	16 (57.1)	13 (81.2)	3 (18.8)	
Acute GvHD (<100 days post transplant) grading				
Absent	19 (67.9)	15 (79.0)	4 (21.0)	0.846
I	3 (10.7)	2 (66.7)	1 (33.3)	
≥II	6 (21.4)	5 (83.3)	1 (16.7)	
Chronic GvHD (≥100 days post transplant) grading[3]				
Absent	23 (92)	18 (78.3)	5 (21.7)	0.369
Mild to severe	2 (8)	1 (50.0)	1 (50.0)	
EBV serostatus				
R+	24 (85.7)	20 (83.3)	4 (16.7)	0.133
R−	4 (14.3)	2 (50.0)	2 (50.0)	

Comparisons between patient-related and transplant-related characteristics and EBV DNAemia were evaluated using chi-square test of independence. Bold *P*-values are statistically significant.
[1]Diamond-Blackfan anemia ($n = 2$); Thalassemia major ($n = 2$); Langerhans cell histiocytosis ($n = 1$); Fanconi anemia ($n = 1$); sickle cell disease ($n = 1$); familial hemophagocytic lymphohistiocytosis ($n = 1$).
[2]Myeloablative conditioning regimen includes both busulfan-based myeloablative regimen and total body irradiation-based myeloablative regimen.
[3]Data available for 25 patients.
ALL, acute lymphoblastic leukemia; SAA, severe aplastic anemia; AML, acute myeloid leukemia; MUD, matched-unrelated donor; ATG, anti-thymocyte immunoglobulin; CMV, cytomegalovirus; GvHD, graft-versus-host disease; R, recipient.

Table 2

Discussion

EBV-PTLD is an uncommon, but frequently fatal, complication after allo-HSCT and prompt diagnosis is necessary owing to the rapid and often disseminated nature of the disease (25–27). In the past, mortality rates from EBV-driven disease were >90%. In fact, its treatment was limited to reduction of immunosuppression, chemotherapy, or unselected donor lymphocytes infusion. Current treatment strategies, such as anti-CD20 monoclonal antibody rituximab and EBV-specific cytotoxic T-lymphocytes therapy, have significantly reduced the risk of death in established EBV-PTLD after SCT (28). In the present study, 28 pediatric allo-HSCT recipients were monitored for EBV infection during the post-transplant period. Virological monitoring of EBV infection was performed for all patients by processing WB samples using a quantitative real-time PCR. EBV DNA quantification in both WB and in PBMC samples was adopted for the patients at higher risk of developing EBV-PTLD (≥10,000 copies/mL WB).

As reported in previous studies (18, 19), we observed that active EBV infection is a frequent event after transplantation: the incidence of EBV DNAemia in our study population was equal to 57.1%. Moreover, high EBV DNAemia levels were observed in 37.5% of the recipients who developed post-transplant EBV infection. We identified that SAA, unrelated donor, the RIC regimen and, to a lesser extent, *in vivo* T-cell depletion with ATG were associated with higher frequencies of EBV infection with high viral load, pointing to the same results recognized by other investigators (24, 26, 27, 29–32). The duration between disease diagnosis and type of treatments before transplant was evaluated (data not shown). In particular, in patients affected from SAA, immunosuppressive therapy is the only alternative therapeutic approach to HSCT and it includes the use of ATG and cyclosporine. Therefore, these patients were intensively treated before allo-HSCT and this could be the reason why we observed a higher incidence of EBV infection with high viral load among these patients. Moreover, the patients with SAA had other risk factors such as unrelated donor, RIC regimen, EBV serology mismatch between donor and recipient, and *in vivo* T-cell depletion with ATG. The higher incidence of high viral load in patients with an unrelated donor could be related to the use of ATG in this group of patients; in fact, the use of ATG has been clearly associated with the risk of both EBV infection and PTLD development (24, 26, 27, 29).

Finally, as reported in other studies (30–32), EBV infection with high viral load appears to be significantly related in children following RIC HSCT, particularly with selective depletion of recipient T cells with ATG relative to B cells. This finding probably reflects the profound immunosuppression following RIC HSCT, together with the incomplete ablation of recipient-derived B cells. In our cohort of patients, we were not able to evaluate the contribution of EBV D/R serology to EBV DNAemia, because donor EBV serology was available for 14 of 24 EBV-seropositive patients (n = 2 D−/R+ and 12 D+/R+) and for only 1 of 4 EBV-seronegative patients (D+/R−). Furthermore, 1 of the 3 remaining EBV-seronegative patients (D?/R−) did not develop post-transplant EBV infection, leading us to assume that the respective donor could be EBV-seronegative. However, it should be noted that all 3 patients with known serological mismatch (D−/R+ and D+/R−) developed a post-transplant EBV infection with high viral load. Therefore, only the recipients' EBV serostatus at the time of transplant was taken into consideration for the analysis and no difference was found between this variable and the EBV DNAemia levels. We suppose that the inability to find the EBV seronegativity of the recipient, associated with an increased risk of developing EBV infection with high viral load, may have been a result of the small number of patients in this group and the lack of information about the EBV status of the respective donors.

Measurement of EBV DNA load by quantitative real-time PCR assays can be a sensitive aid to PTLD diagnosis, but unfortunately it is not always specific for disease onset (3). In general, assays using PBMCs are the most sensitive and predict EBV disease at the earliest time, but owing to technical aspects, it would be preferable to test EBV DNA load in WB samples (3, 33). In agreement with previous studies (34, 35), we obtained a significant correlation between EBV DNA levels in WB and PBMC samples.

Moreover, a similar kinetics of EBV DNA load in the 2 different blood compartments of the pediatric patients was observed: peak DNA levels were reached simultaneously and the times of DNA clearance were comparable. In addition, both specimen types appeared to be equally informative to assess the risk of patients to develop PTLD in the clinical setting. In fact, in all patients, EBV infection was managed with reduction of immune suppression or with the use of anti-CD20 monoclonal antibody rituximab, at peak DNA levels that were reached simultaneously in both the 2 blood compartments. Therefore, among the study population, on the basis of EBV DNA levels detected in WB samples, we were able to identify 3 patients (10.7%) eligible for

preemptive therapy and 1 patient (3.5%) who needed early treatment for probable EBV disease. In patients, the first-line treatment for EBV-related disease was effective in controlling viral proliferation and avoiding progression into EBV-PTLD. In fact, in our study population none of the patients developed EBV-PTLD.

In conclusion, the present study suggests that close EBV DNAemia monitoring may be a useful strategy to control EBV-related PTLD in high-risk patients. In fact, WB proved to be a suitable clinical specimen to monitor EBV DNA load after allo-HSCT for the management of post-transplant EBV infection and PTLD prevention. Moreover, this study confirms the clinical parameters associated with an increased risk of developing EBV infection with high viral load. The presence of the risk factors may be useful to identify patients who require closer virological monitoring.

EBV DNA monitoring is essential for managing EBV infection, but it does not provide information regarding a patient's immunological response against EBV replication. A prospective immunological monitoring with EliSpot assay combined with virological monitoring is in progress to assess if this monitoring strategy could improve the managing of EBV infection and the clinical decision-making in allo-HSTC recipients.

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